Patterns of Nitrogen Excretion by Embryonic Softshell Turtles (*Trionyx spiniferus*) Developing in Cleidoic Eggs

Abstract. *Embryonic softshell turtles develop inside eggs exchanging little, if any, liquid water with the nest environment. Despite the consequent restriction on availability of water to support development, embryos convert most of the ammonia released in catabolism of proteins into soluble urea rather than insoluble urate, and thereby commit a portion of their limited reserve of water to serve as solvent for metabolic wastes. This finding is contrary to expectations from prior studies of cleidoic eggs.*

One of the most compelling generalizations of comparative biochemistry and physiology concerns the pattern of nitrogen excretion by embryos of oviparous, terrestrial vertebrates. According to the widely held view (*1*), embryos of birds and certain reptiles develop inside cleidoic eggs (*2*), which have relatively thick, calcareous shells that protect enclosed embryos against the dangers of desiccation accompanying development in terrestrial environments. Only respiratory gases and water vapor normally are exchanged between the interior of these eggs and their surroundings (*2*). Because no water in liquid form is taken up from the environment, embryos are forced to subsist on whatever water is present in eggs at oviposition plus small quantities of water formed metabolically by the embryos themselves. A major physiological adaptation of these embryos entails converting ammonia released in catabolism of proteins into uric acid (or urate) that is precipitated in the chorioallantois. In precipitated form, uric acid does not occupy osmotic space, so that solvent capacity of the limited reservoir of water is conserved for more direct support of embyronic metabolism. Thus, synthesis of uric acid from ammonia is a means of preventing accumulation of a toxic material inside eggs while simultaneously conserving water that would be required as a solvent if more soluble wastes, such as urea, were to be formed (*2, 3*).

Most evidence on the cleiodic egg comes from studies of avian eggs, and few studies have been performed on reptilian embryos. We studied the pattern of nitrogen excretion by embryos of softshell turtles (*Trionyx spiniferus*), a species producing eggs with rigid, calcareous shells (*4*) that are only slightly more permeable to water vapor than are the eggs of birds (*5*). Little, if any, liquid water passes between the interior of these eggs and the nest environment (*6*), so that the eggs are functionally cleidoic in Needham's definition (*2*). Contrary to what would be expected from the preceding account of cleidoic eggs, developing embryos of softshell turtles excrete large quantities of waste nitrogen in the form of urea and lesser amounts in the form of ammonia; both urea and ammonia are soluble substances that occupy osmotic space. Little waste nitrogen is excreted as urate, and none of the detectable urate is in an insoluble form.

Eggs were collected from two natural nests (*7*) and brought to the laboratory where they were half-buried in moistened vermiculite and incubated at 29°C (*8*). On days 36, 44, 48, and 52 of an incubation 55 to 56 days in duration, two eggs from each clutch were homogenized separately in approximately 25 ml of distilled water. After centrifugation, the supernatant was transferred to a volumetric flask, and the pellet was resuspended in water and centrifuged again. The wash was added to the volumetric flask, which was then brought to volume. Samples of this material were used to determine concentrations of ammonia and urea by the Berthelot procedure (*9*), and the concentration of soluble urate was measured by the reduction of phosphotungstate (*10*). The original pellet was then suspended in 25 ml of 0.4 percent lithium carbonate to dissolve precipitated urate (*11, 12*), after which the suspension was centrifuged and the supernatant was transferred to a volumetric flask. The flask was brought to volume with distilled water, and the concentration of urate in this fraction was estimated by the phosphotungstate procedure (*10*). Total amounts of nitrogen in the various forms were then computed for each egg.

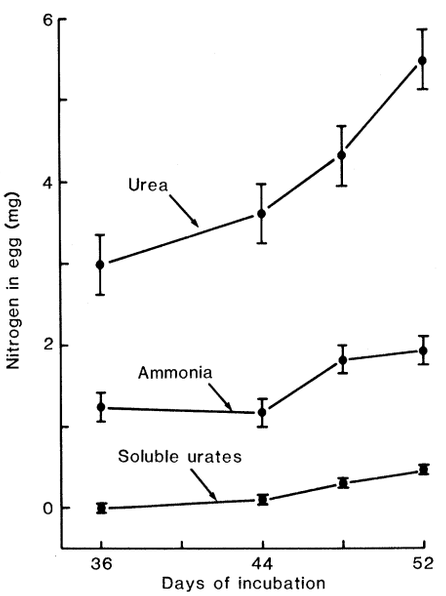

Fig. 1. Amounts of nitrogen excreted in the form of ammonia, urea, and soluble urate by developing embryos of softshell turtles. Insoluble urate did not account for measurable quantities of excretory nitrogen. Each point represents the mean of four measurements, and the error bar represents the least significant difference (*20*) for multiple comparisons ($\alpha = 0.05$) of means for a particular substance.

Eggs incubated for 36 days contained nearly three times more urea-nitrogen than ammonia-nitrogen (Fig. 1), but neither soluble nor insoluble urate was present in detectable amounts. Urea and ammonia continued to accumulate in eggs between days 36 and 52 and accounted for 70 and 24 percent, respectively, of the nitrogenous wastes present in eggs prior to hatching (Fig. 1). Soluble urate was detectable by day 44 and continued to accumulate thereafter, but only 6 percent of the waste nitrogen was present in this form on day 52 (Fig. 1). Insoluble urate never accounted for measurable amounts of excretory nitrogen, and we never noted accumulations in the chorioallantois of the white crystalline material that is so characteristic of avian eggs nearing the end of incubation. Although it is likely that the small quantities of soluble urate were produced in catabolism of purines rather than proteins, it nonetheless is apparent that embryos of softshell turtles are ureotelic rather than uricotelic (that is, they detoxify most of the ammonia present by converting it to urea rather than to uric acid).

Embryos of snakes (*13*), other species of turtles (*12, 14*), and crocodilians (*15*) also detoxify ammonia released in protein catabolism by converting it to urea. The snakes and turtles, however, produce flexible-shelled eggs that are capable of imbibing large quantities of water from the environment (*16*), so that embryos of these reptiles have access to larger amounts of water during development than are generally available to embryos developing in truly cleidoic eggs. Even the hard-shelled eggs of crocodilians may absorb water from the environment (*17*). Thus, it can be argued that embryos of these several reptiles have retained ureotely for detoxifying ammonia simply because there has not been intense natural selection for mechanisms to conserve water for direct support of metabolism.

The results of our study show that

ureotelic metabolism can occur even among embryos developing in fully cleidoic eggs, which do not take up supplemental water from the environment. Retention of ureotely in this case cannot be explained by suggesting that embryonic softshell turtles lack the biochemical preadaptations necessary for uricotely (*18*) because they have the capacity to form urate (Fig. 1). Thus, our results raise the possibility that uricotely is not a necessary outcome of natural selection for mechanisms to conserve water during embryonic development (*19*) and indicate a need to reassess the adaptive significance of uricotely among embryos of other terrestrial vertebrates.

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2. J. Needham [*Chemical Embryology* (Cambridge Univ. Press, Cambridge, 1931), p. 1615] characterized the cleidoic egg as "a closed box, with walls which can only be penetrated by matter in the gaseous state."
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7. Eggs were collected on 23 and 24 June 1982 from natural nests located on sandbars in the South Platte River, near Crook, Logan County, Colorado. White spots had not formed on the uppermost surfaces of the eggs, indicating that nests were only 1 to 3 days old at the time eggs were collected.
8. Half the eggs were incubated on a wet substrate (water potential of −150 kPa), and the rest were incubated on a dry substrate (−800 kPa). See G. C. Packard *et al.* [*Physiol. Zool.* **54**, 165 (1981)] for full details on methods. Because analyses of variance indicated that different substrates for incubating eggs had no statistically significant effect on amounts of nitrogen excreted in the form of ammonia ($P > 0.81$ for main effect and interaction), urea ($P > 0.57$), and soluble urate ($P > 0.25$), data for the two treatments were pooled for presentation here.
9. Ammonia-nitrogen and urea-nitrogen were measured by a colorimetric procedure based on the methods of J. K. Fawcett and J. E. Scott [*J. Clin. Pathol.* **13**, 156 (1960)] and of A. L. Chaney and E. P. Marbach [*Clin. Chem. (Winston-Salem, N.C.)* **8**, 130 (1962)], as detailed in *Sigma Tech. Bull. 640* (Sigma Chemical, St. Louis, revised October 1974). Readings of absorbance were taken at 570 nm with a Bausch & Lomb Spectronic 21 digital spectrophotometer.
10. Uric acid was measured by a colorimetric procedure based on the methods of M. B. Blauch and F. C. Koch [*J. Biol. Chem.* **130**, 443 (1939)] and of R. J. Henry *et al.* [*Am. J. Clin. Pathol.* **28**, 152 (1957)], as detailed in *Sigma Tech. Bull. 680* (Sigma Chemical, St. Louis, revised June 1974). The procedure entails digestion by uricase to correct for interfering substances. Absorbances were read at 650 nm.
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21. Turtle eggs were collected under authority of permit 82-033 issued by the Colorado Division of Wildlife. We are grateful to R. D. Gettinger, G. L. Paukstis, and W. H. N. Gutzke for helping us to locate the eggs used in this study. We also thank T. J. Boardman for assistance with statistical analysis of the data and K. Jee for executing the line drawing. Supported in part by NSF grant DEB 79-11546.

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Burst Discharge in Mammalian Neuroendocrine Cells Involves an Intrinsic Regenerative Mechanism

Abstract. *Intracellular recordings from mammalian neuroendocrine cells showed that steady, injected currents can modify and block periodic spike bursts previously associated with increased neurohormone release. Spike afterpotentials could sum to form plateau potentials, which generated bursts and did not depend on axonal conduction or chemical synapses. Therefore, bursting involves a spike-dependent, positive-feedback mechanism endogenous to single neuroendocrine cells.*

Neuroendocrine cells, whose electrical activity causes secretion of peptides into the nervous and circulatory systems, are vital in the regulation of such diverse bodily functions as reproduction (*1*), endocrine control (*2*), and maintaining salt and water balance. The cells that secrete vasopressin and oxytocin have been the primary model, particularly in mammals, for electrophysiological (*1*) and biochemical (*3*) studies of neuroendocrine systems. In vivo extracellular

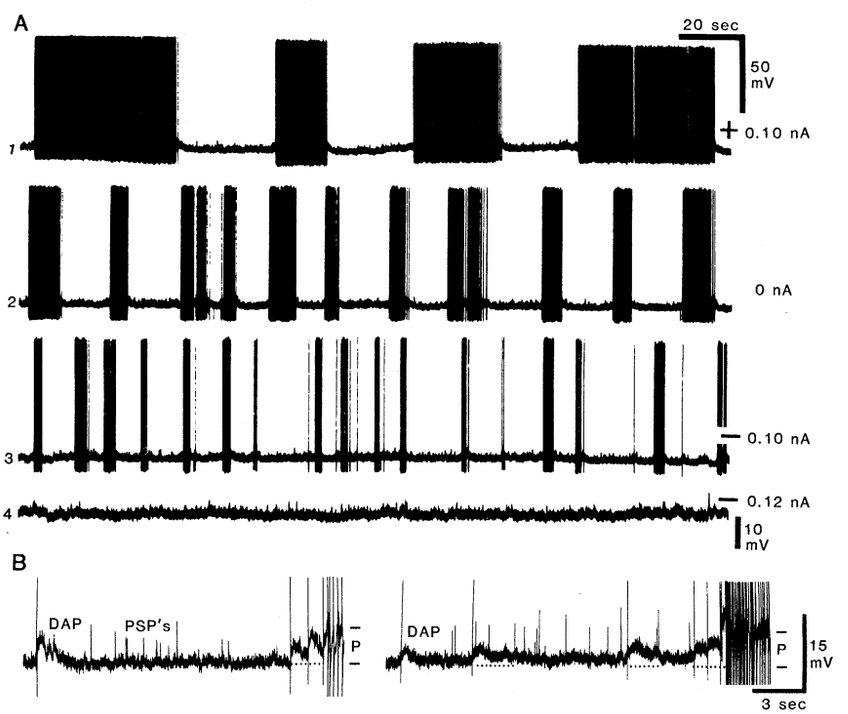

Fig. 1. (A) Alteration of burst length in a phasic burster by steady changes in membrane potential from injected current. Membrane depolarization (trace 1) promoted longer bursts with higher spike frequency than observed at resting potential (trace 2). Conversely, membrane hyperpolarization caused shorter bursts with lower intraburst spike frequency (trace 3). Stronger hyperpolarizing currents (trace 4) blocked the slow underlying depolarization and revealed postsynaptic potentials (*PSP's*) that appeared random. Neuronal input resistance was ~ 120 megohms. (B) Depolarizing afterpotentials and plateau potentials in two phasic bursters. A DAP followed each action potential during the period between bursts. The DAP's could summate, leading to a plateau potential (*P*) and an overriding spike burst. Spike peaks are not shown. Input resistance was ~ 190 megohms (left) and ~ 200 megohms (right). Small depolarizations are excitatory postsynaptic potentials.