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ACCLIMATION OF THE EURYHALINE TOAD *BUFO VIRIDIS* TO HYPEROSMOTIC SOLUTION (NaCl, UREA AND MANNITOL)

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SUMMARY

1. *Bufo viridis* were acclimated to hyperosmotic solutions of NaCl, urea or mannitol.

2. The toads could not be acclimated to mannitol solutions of osmotic strength higher than 300 mosmol kg^{-1} H_2O, but could be acclimated easily to NaCl and urea higher than 500 mosmol kg^{-1} H_2O.

3. Water uptake diminished under mannitol acclimation while the apparent osmotic permeability under NaCl and urea acclimation increased.

4. Urea and sodium influx across the isolated skin changed inversely upon hyperosmotic acclimation, but they did not seem to depend on one another.

5. The adaptational advantages of the observed changes are discussed.

INTRODUCTION

Urea appears to play a critical role in adaptation of amphibians to a terrestrial environment (Bentley, 1966; McClanahan, 1967; Degani, Silankove & Shkolnik, 1981), and in adaptation of euryhaline species to high salinities (see Balinsky, 1981 for a recent review). However, little is known about the physiological mechanisms of urea retention and accumulation under changing environmental conditions. *Bufo viridis*, which is a terrestrial anuran, is one of the most adaptable species (Stoicovici & Pora, 1951; Gordon, 1962; Tercafs & Shoeffeniels, 1962; Katz, 1973*a*). We have studied the acclimation of this species to hyperosmotic solutions of NaCl, urea or mannitol. We found that the urea and sodium transport pathways, and the cutaneous water permeability, responded selectively to the various acclimation solutions, such as to reduce sodium entry and to enhance urea accumulation.

MATERIALS AND METHODS

Toads of both sexes were collected in Israel at the same locality (Maale-Ephraim) and were kept in the laboratory at 21 °C. They were acclimated to the hyperosmotic solutions by keeping them for 3–4 days in a concentration of 200 mosmol kg^{-1} H_2O for each solution before being transferred to the 400 mosmol kg^{-1} H_2O solutions. The

Key words: Urea influx, water uptake, I_{sc}, amiloride, drinking.

toads were kept at least 10 days in the experimental solutions, which were replaced daily, and they were not fed during the experimental period. Water uptake was determined from the weight change of individually caged toads (Katz, 1974) to the nearest 0·01 g. Surface area was calculated from the empirical equation $A(cm^2) = W^{6·3} \times 6·3$ (W = weight in grams). Blood was collected from the heart immediately after decapitation, into heparinized polyethylene tubes. The blood was centrifuged, the haematocrit was determined and the plasma was used for chemical analyses. Samples were taken from the liver, sartorius muscle, heart and the skin. They were blotted on Whatman No. 2 filter-paper and dried to constant weight at 90 °C. Osmolality was determined on 50-μl samples with a semimicro osmometer (Knauer, Berlin). Chloride was titrated with a Radiometer CMT 10 chloridometer. Sodium and potassium were determined by flame photometry and urea was determined according to the method of Foster & Hochholzer (1972). Urea fluxes were measured using ^{14}C-urea for pieces of abdominal skin ($A = 3·14\,cm^2$) mounted in Perspex chambers. Sodium transport was estimated from the short-circuit current (I_{sc}), which was measured by a voltage clamp device. The solution in the *in vitro* studies had the following composition (mmol l^{-1}): NaCl, 115; KCl, 3; $CaCl_2$, 1; $MgSO_4$, 0·5; mannitol, 25; urea, 5; Tris, 0·5; glucose, 0·5; osmolality 270 mosmol kg^{-1} H_2O; pH, 8·0; High osmolality solution (530 mosmol kg^{-1}) had in addition 285 mmol l^{-1} of mannitol. Syntocinon was from Sandoz and amiloride from Merck, Sharp & Dohme. Student's *t*-test was used for statistical analysis.

RESULTS

While acclimation of the toads to NaCl and urea solutions was easy and eventually they could be kept for over 2 weeks at 500 mosmol kg^{-1} H_2O without mortality, weight loss and mortality occurred in the mannitol solution, beginning at osmolalities higher than 300 mosmol kg^{-1} H_2O. We therefore chose to study the acclimation to 400 mosmol kg^{-1} H_2O for all three solutes. During the course of acclimation through 200 mosmol kg^{-1} H_2O to 400 mosmol kg^{-1} H_2O the change in weight of toads in the urea solution was scarcely different from that of toads in tap water (Fig. 1). In 200 mosmol kg^{-1} H_2O NaCl solution the toads gained some weight (up to 5 %); there was a rapid loss upon transfer to 400 mosmol kg^{-1} H_2O which was regained in the following 3–4 days. In mannitol, high mortality occurred and some 50 % of the toads died at an osmolality of 400 mosmol kg^{-1} H_2O. They lost some 15 % of their initial weight upon transfer from 200 mosmol kg^{-1} H_2O into the 400 mosmol kg^{-1} H_2O solution which they did not recover. Addition of NaCl (25 mmol l^{-1}) did not improve the ability of the toads to acclimate in 400 mosmol kg^{-1} H_2O mannitol.

Water uptake, which was some 1 g 100 cm^{-2} h^{-1} in water-acclimated toads, was drastically reduced in the mannitol-acclimated group. Water uptake in the NaCl-acclimated toads was quite similar to that for urea acclimation, ranging from 1/3 to 1/2 of the rate in water-acclimated toads (Fig. 2). Toads drank occasionally under both the urea and NaCl acclimation conditions. However, this was irregular and was not quantified.

The osmolality of the blood of the toads was highest in the NaCl-acclimated grou

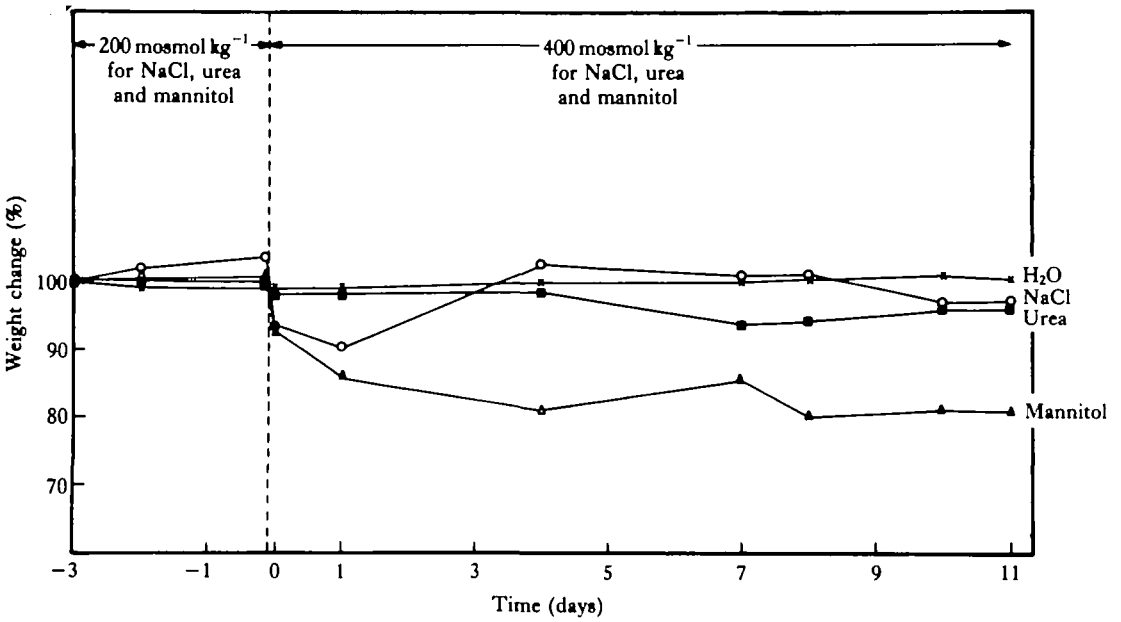

Fig. 1. Time course of the relative weight change of toads (*Bufo viridis*) during acclimation to various solutes. Four to six toads weighing about 45 g each were pooled and weighed together. ○, NaCl; ■, urea, △, mannitol. (x———x) Shows the weight of toads which were maintained in tap water. (Penicillin and streptomycin were added to the urea solution to prevent bacterial development.)

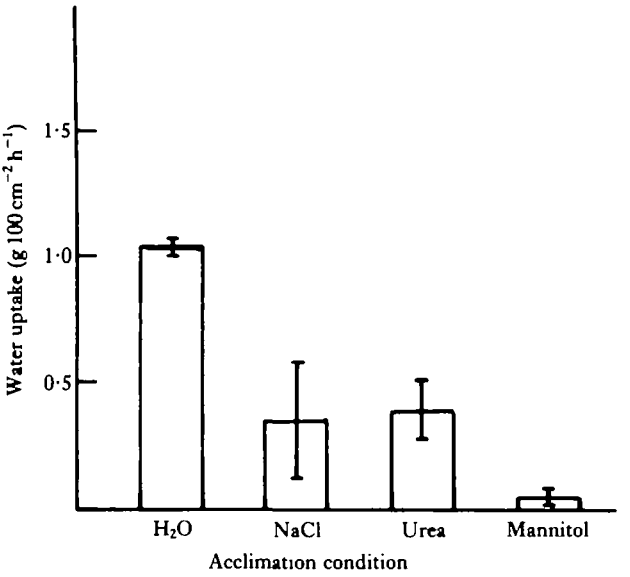

Fig. 2. *In vivo* water uptake of toads (*Bufo viridis*) which were acclimated to 400 mosmol kg^{-1} H_2O of various solutes for 10–14 days. Water uptake was determined in the solution of acclimation under each condition. The water uptake of the 'NaCl' and 'urea' groups do not differ from one another. However, they differ significantly from the 'H_2O' group ($P > 0{\cdot}05$) and the mannitol group ($P > 0{\cdot}01$). Mean ± S.E. of four to five animals under each condition.

and lowest in the 'mannitol' group (Table 1). The 'urea' group had the lowest concentration of NaCl in the plasma. It should be pointed out here that the osmolality and the composition of the blood of the toads in all groups was different in the summer as compared with the winter. The osmolality may become as much as 100 mosmol kg^{-1} H_2O higher in the summer of which over 50 % could be attributed to urea. This observation substantiates earlier observations (Katz, 1973*a*). The haematocrit of the blood, which increased by some 25 % in the 'NaCl' and 'urea' groups was more than doubled in mannitol (Table 1), as a result of considerable water loss under this condition. This loss was also reflected in the water content of the tissues and carcasses of the toads of the various groups (Table 2). The water content of the carcass was determined after removing the fat bodies and the gonads. The toads which had been kept in mannitol were dehydrated considerably when compared to all other groups.

Table 1. *Composition of the plasma of* Bufo viridis *which were maintained in tap water or acclimated to NaCl, urea and mannitol solutions of 400 mosmol* kg^{-1} H_2O

Solution of acclimation	H_2O	NaCl	Urea	Mannitol
Haematocrit (%)	26·7 ± 1·4	38·5 ± 8·5	36·8 ± 5·4	59·3 ± 5·0
Osmolality (mosmol kg^{-1})	284 ± 5	494 ± 12	440 ± 14	404 ± 11
Na^+ (mmol l^{-1})	119 ± 5	170 ± 5	114 ± 7	131 ± 6
K^+ (mmol l^{-1})	4 ± 0·5	4 ± 0·5	4 ± 0·5	6 ± 2
Cl^- (mmol l^{-1})	100 ± 10	157 ± 7	108 ± 5	120 ± 8
Urea (mmol l^{-1})	30 ± 4	125 ± 8	155 ± 8	75 ± 7
N	4	5	5	4

Mean ± S.E. *N* = number of toads.

Table 2. *Water content (per cent of fresh weight) of the carcass, sartorius muscle and the skin of* Bufo viridis *under acclimation to various solutes at 400 mosmol* kg^{-1} H_2O

Solution of acclimation	H_2O (I)	NaCl (II)	Urea (III)	Mannitol (IV)
1. Carcass	74·6 ± 1·0	73·7 ± 1·0	70·7 ± 1·5	68·4 ± 1·4
2. Sartorius muscle	77·0 ± 1·1	73·7 ± 0·3	74·8 ± 0·6	72·8 ± 0·7
3. Skin	72·2 ± 1·4	70·0 ± 1·4	72·1 ± 0·8	67·7 ± 1·4
N	4	4	5	4
Comparison				
Carcass	I:II	II:III	III:I	IV:I
P	<0·01	<0·025	<0·001	>0·025
Sartorius muscle	I:II			IV:I
P	>0·2			>0·1
Skin	I:II			IV:I
P	<0·05			<0·2

Mean ± S.E. *N* = number of toads.

Flux measurements were made on the abdominal skins under open circuit conditions with identical solutions (either low or high osmolality) containing 5 mmol l^{-1} urea on both sides. Urea efflux (determined on pieces of skin from seven differe

Table 3. *Urea influx across isolated abdominal skin of* Bufo viridis *which were maintained in tap water or acclimated in 400 mosmol kg^{-1} H_2O of either NaCl, urea or mannitol*

Solution of acclimation	H_2O (I)	NaCl (II)	Urea (III)	Mannitol (IV)
Influx (μmol cm^{-2} h^{-1})	19·0 ± 7·6	187·1 ± 71·9	27·3 ± 5·2	73·3 ± 22·5
N	6	6	9	9
Comparison	I:III	II:IV		IV:I
P	>0·2	>0·05		<0·01

The bathing solutions contained 5 mmol l^{-1} urea on both sides.
Mean ± S.E. *N* = number of toads.

Table 4. *Electrical properties and amiloride-blockable stimulated (syntocinon 100 mU ml^{-1}) short-circuit current of isolated abdominal skin of* Bufo viridis *which were maintained in tap water or acclimated in 400 mosmol kg^{-1} H_2O of either NaCl, urea or mannitol*

Solution of acclimation	H_2O (I)	NaCl (II)	Urea (III)	Mannitol (IV)
PD (mV)	8·2 ± 4·3	11·0 ± 6·5	20·1 ± 6·0	0·1 ± 0·9
I_{sc} (μA cm^{-2})	−4·6 ± 1·5	−0·5 ± 0·3	−9·8 ± 3·7	−2·8 ± 0·9
R (Ωcm^2)	1215 ± 379	20 531 ± 8162	2459 ± 407	3806 ± 1184
Amiloride-blockable Na transport (μA cm^{-2})	12·0 ± 3·0	0·9 ± 0·4	20·8 ± 3·6	2·4 ± 0·6
N	4	4	5	3
Comparison	I:II	II:IV	III:I	I:IV
P	<0·001	<0·005	>0·05	<0·001

Mean ± S.E. *N* = number of toads.

animals was 11·1 ± 1·8 μmol cm^{-2} h^{-1} (mean ± S.E.), and was not different in the various acclimation conditions. The influx of urea on the other hand changed in a characteristic manner (Table 3), and was more than double in the mannitol-acclimated group compared to both the water- and urea-acclimated groups. The influxes in the latter groups were similar to one another, with the highest rate of urea influx seen in the 'NaCl' group. Table 4 summarizes the electrical properties of the skins of the toads: these measurements were taken after the urea measurements were completed. The lowest electrical resistance was found in the skins of 'H_2O' toads; it became over twice as high in the 'urea' and 'mannitol' groups, and over 10 times as high in the skins of the NaCl-acclimated toads. At the end, syntocinon (100 mU ml^{-1}), was applied to the inner bathing solution, the stimulated I_{sc} was recorded after 20 min, and amiloride (50 μmol l^{-1}) was added to the outer bathing solution. The amiloride-blockable sodium transport is highest in skins of urea-acclimated toads (Table 4). It was reduced significantly in the 'mannitol' group and nearly eliminated in the skin from NaCl-acclimated toads.

DISCUSSION

The present study shows that *B. viridis*, which is an extremely euryhaline toad, cannot be acclimated readily to mannitol solutions at osmolalities higher than 300 mosmol kg^{-1} H_2O. It can acclimate easily, however, in urea and NaCl solutions at osmolalities higher even than 500 mosmol kg^{-1} H_2O. In fact, this species can be acclimated to NaCl solutions up to 800 mosmol kg^{-1} (Katz, 1973*a*) and tolerates severe dehydrating conditions (Degani *et al.* 1981). The high haematocrit and the reduced water content of the tissues in the mannitol-acclimated group indicate that the toads were considerably dehydrated.

The most significant difference between the three acclimation groups is found in the rate of water uptake across the skin. The rate in tap water was about twice that in urea- and NaCl-acclimated toads, and about 10 times that in mannitol-acclimated toads.

Since the osmotic gradient across the skin differed little between the three acclimation groups, it follows that the apparent osmotic permeability (P_{osm}) of the skin in the NaCl- and urea-acclimated toads had increased considerably; this was suggested earlier on the basis of *in vitro* studies (Katz & Weissberg, 1971; Katz, 1973*b*). In the mannitol-acclimated toads, on the other hand, the P_{osm} had been greatly reduced, as was observed previously in toads acclimated to 230 mosmol kg^{-1} sucrose (Katz, 1973*b*).

The osmolality and the composition of the plasma in the three acclimation groups were somewhat different from one another. This suggests different osmoregulatory capacities of the toads in the isosmolar solutions of the various solutes. Plasma osmolality was highest in the NaCl-acclimated toads and lowest under mannitol acclimation. This was also reflected in the differential changes of the concentrations of electrolytes and urea in the plasma. Similar results were obtained by Funkhouser & Goldstein (1973), and by Schrock, Bauer, Merkle & Hanke (1980) in *Xenopus laevis* acclimated in mannitol solutions isosmotic to the blood or 300 mosmol kg^{-1} H_2O.

Increased urea concentration in the plasma seems to be an essential feature of hyperosmotic acclimation, common to all euryhaline amphibians (Balinsky, 1981). The mechanisms for urea acclimation include reduced renal filtration and increased reabsorption in the kidneys (McBean & Goldstein, 1970*a*), urinary bladder and the skin (Katz, Garcia-Romeu, Masoni & Isaia, 1981), and accelerated synthesis by the urea cycle enzymes (McBean & Goldstein, 1970*b*). These mechanisms may be activated differentially in the various species and also in the same species. We show in this study that urea- and sodium-transport pathways in the skin are modulated according to environmental stress. Thus urea influx (active uptake) across the skin has its lowest rate in the skins of both water- and urea-acclimated toads, which had on the other hand higher rates of sodium uptake as estimated from the amiloride-blockable short-circuit current (I_{sc}). In skins of the mannitol- and NaCl-acclimated toads, higher urea influx was observed, while I_{sc} was greatly diminished. Although it is tempting to speculate on a possible interrelationship of the urea and sodium influxes, these processes do not seem to be dependent on one another. This conclusion is supported by an experiment where toads were immersed in tap water with amiloride (100 μmol l^{-1}) for 10 days without any effect on urea influx across the isolated skin

(unpublished observations). The changes in these transport pathways are of adaptational advantage in terms of the response to environmental challenge. The urea uptake mechanism is induced under hyperosmotic stress, but not in a urea-rich environment, while sodium uptake is blocked in an environment which is rich in NaCl.

The kidney should contribute significantly to osmoregulation under changing environmental conditions, and we have observed differences in the volume and composition of the urine under the various acclimation conditions.

We have found in this study that *Bufo viridis* can be acclimated to great increases in the osmolalities of the external environment if these increases are brought about by NaCl or urea. However, the toad is unable to deal with external hypertonicity when this is produced by an impermeant solute like mannitol. This is apparently because the necessary osmoregulatory mechanisms fail to increase and maintain the blood osmolality hypertonic to the external solution.

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