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THE EFFECT OF SALT ADAPTATION AND AMILORIDE ON THE *IN VIVO* ACID–BASE STATUS OF THE EURYHALINE TOAD *BUFO VIRIDIS*

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SUMMARY

1. The acid–base status of the blood of the toad *Bufo viridis* was studied during adaptation to high salinity and in tap water containing amiloride.

2. Both salt adaptation and immersion for 2–3 days in 5×10^{-4} M amiloride in tap water resulted in a decrease in blood pH (from $7{\cdot}720 \pm 0{\cdot}026$ in tap water to $7{\cdot}456 \pm 0{\cdot}051$ in 500 mOsm NaCl-adapted toads; mean ± S.E.), and a simultaneous decrease in the concentration of HCO_3^- (from $17{\cdot}8 \pm 1{\cdot}4$ in tap water to $9{\cdot}5 \pm 1{\cdot}2$ in salt-adapted toads).

3. *In vitro* determination of Na^+/H^+ exchange across the skin showed a 1:1 relation in skins from tap-water-adapted toads; this exchange was inhibited by amiloride. H^+ secretion was abolished in skins from salt-adapted toads and the uptake of sodium was reduced.

INTRODUCTION

The euryhaline toad *Bufo viridis* can be adapted to extremely high salinity (Stoicovici & Pora, 1951; Tercafs & Schoffeniels, 1962; Gordon, 1962; Katz, 1973). Under these conditions there is a great increase in the osmolarity of the blood; both the concentration of NaCl and of urea increase, and there are marked changes in transport properties of the skin (Katz, 1975). The control of acid–base status in amphibia is different from that in higher vertebrates – notably mammals; amphibian kidneys are much less effective (Yoshimura *et al.* 1961), and the urinary bladder may play a role in the acid–base regulation in some species or subspecies, but not in others (Ziegler, Ludens & Fanestill, 1974). The skin, which is endowed with an Na^+/H^+ exchange mechanism operating under open-circuit conditions (Garcia-Romeu, 1971; Ehrenfeld & Garcia-Romeu, 1977), may also participate in amphibian acid–base regulation. This study is concerned with the *in vivo* acid–base status of the blood in the toad *Bufo viridis* during adaptation to high salinity, and compares this with the effect of amiloride. A preliminary report has been published (Katz, 1979).

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MATERIALS AND METHODS

Toads (*Bufo viridis*) were collected in Jerusalem (Israel) and were kept outdoors with free access to tap water. They were fed twice a week with maggots. During the experiments the toads were kept in the laboratory (19–25 °C) and were not fed. Salt adaptation was carried out gradually (Katz, 1973). Toads were immersed to a depth of 2–3 cm, first in NaCl solution of 230 mOsm for 5–7 days and then directly into a 500 mOsm solution. For the collection of blood a permanent cannula was implanted into the dorsal artery as detailed elsewhere (Katz, 1980). Blood was collected anaerobically from unrestrained animals and the pH, P_{CO_2} and P_{O_2} were determined immediately, using a Radiometer BMS 3 MK 2 blood microsystem and Radiometer electrodes, attached to PHM 73 pH/blood gas monitor. All electrodes were recalibrated after each measurement and readings were corrected accordingly. Osmolarity was determined on a Knauer (Berlin) semimicro osmometer, and chloride on a Radiometer CMT 10 chloride titrator. Na^+/H^+ exchange was determined *in vitro* on abdominal skins under open-circuit conditions with low (2 mM) sodium on the outside, according to the method of Ehrenfeld & Garcia-Romeu (1977). The internal solution contained (in mM): NaCl, 83; $NaHCO_3$, 24; Na_2HPO_4, 4; KCl, 2·5; KH_2PO_4, 1·3; $CaCl_2$, 4; $MgSO_4$, 4; glucose, 11, gassed with 95% O_2/5% CO_2 mixture, pH 7·4. In the external solution there was only 2 mM NaCl buffered with imidiazole (2 mM) to pH 7·4, and the solution was gassed with air. The unidirectional influx of sodium was determined with ^{22}Na added to the mucosal side, and hydrogen net fluxes were obtained from the changes in H^+ concentration on each side during the flux period. H^+ was titrated with 2 mM NaOH in 2 ml samples with a Radiometer ATSI/TTA61 autopipetting titration system, after 30 min bubbling air to eliminate respiratory CO_2. Potential difference was measured with calomel electrodes through agar bridges. Amiloride was from Merck, Sharp & Dohme. Student's *t* test was used for statistical analysis.

RESULTS

Fig. 1 shows the time course of changes in the pH, HCO_3^-, and osmolarity of the blood in 4 toads during adaptation to high salinity. There is a significant reduction in the pH and HCO_3^-, while the osmolarity nearly doubled, after 6 days in 500 mOsm NaCl (the toads can live under these conditions for over a month; Katz, 1973). Transferring the toads back to tap water resulted in a rapid return of these parameters to their previous control values. Table 1 summarizes the pH, blood gases and the osmolarity of the blood in toads which were adapted to high salinities. It should be pointed out that the control group had a free access to tap water, while the others were immersed 2–3 cm in the solution. This has been found to have no effect on the acid–base status of the blood (Katz, 1980). There is a considerable acidification of the blood and a simultaneous reduction in the concentration of HCO_3^- at the high salt conditions (500 mOsm), characteristic of metabolic acidosis. P_{CO_2} did not change, and neither was there a change in the P_{O_2}. The osmolarity of the plasma, on the other hand, increased from 370 to 565 mOsm/kg H_2O, and the concentration of the chloride doubled. A calculation of the ionic contribution to the osmola[illegible]

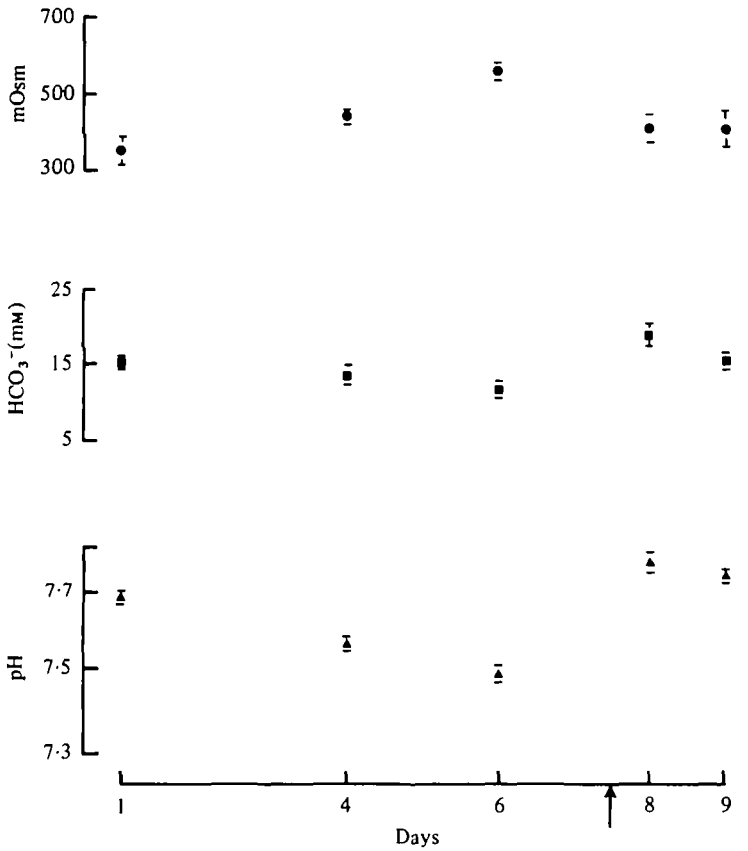

Fig 1 The time course of changes in the pH, HCO_3^-, and osmolarity (mOsm), in four toads during adaptation to high salinity (500 mOsm NaCl). Mean ± S.E. The toads were adapted for over two weeks in 230 mOsm NaCl before transferring them (day No. 1 – after blood sampling), to the 500 mOsm solution. On the seventh day (arrowed), the toads were returned to tap water. Average weight of the toads was 31·8 ± 1·1 g (mean ± S.E.). t = 21 °C (winter 1978).

in Table 1 reveals that there is always some 20–70 mM of other osmolyte, which is contributed by the urea in the blood (Katz, 1973). Two days after transference back to tap water, all variables returned to their previous control values.

It is now established that sodium transport is markedly diminished under conditions of adaptation to high salinity (Katz, 1975) and that this may result in a diminution of H^+ secretion (Garcia-Romeu, 1971). Table 2 summarizes *in vitro* measurements of the Na^+/H^+ exchange across the isolated skin of the toad using the method of Ehrenfeld & Garcia-Romeu (1977). There was a linear and quite significant (r = 0·83) correlation between the net fluxes of these two cations, in skins from toads in deionized water:

$J_{net}H^+ = (-29 \pm 26) - (0{\cdot}99 \pm 0{\cdot}20)\ J_{net}Na^+$ (mean ± S.E.M. from 14 separate skins).

This gives a 1:1 exchange ratio between the two cations under these conditions. Amiloride, which blocks sodium transport across the skin (Eigler, Kelter & Renner, [illegible]7), had a marked effect on the two fluxes in skins from toads adapted in deionized

Table 1. *The effect of salt adaptation on blood data analyses in the toad* Bufo viridis

(Weight of the animals was 31 ± 3 g and the haematocrit was 17·2 ± 6·6 %. t = 21 °C (1978). The toads spent 7 days in 230 mOsm and 6–11 days in 500 mOsm NaCl. The results are from the last day in each experimental condition. Mean ± S.E. Number of toads in parentheses.)*

	pH	P_{CO_2} (mmHg)	HCO_3^- mM	P_{O_2} (mmHg)	Osmolarity (mOsm)	Cl^- mM	Haematocrit (%)
1. Control† (10) (free access to tap water)	7·720 ± 0·026	10·5 ± 0·7	17·8 ± 1·4	97 ± 5	374 ± 7	133 ± 3	18·1 ± 0·8
2. 230 mOsm (10)	7·680 ± 0·007	9·2 ± 0·3	14·1 ± 0·3	95 ± 2	347 ± 3	132 ± 3	18·0 ± 0·7
3. 500 mOsm (5)	7·456 ± 0·051	10·7 ± 0·7	9·5 ± 1·2	92 ± 8	563 ± 12	244 ± 7	19·0 ± 4·5
4. Control‡ (5)	7·783 ± 0·015	10·1 ± 0·5	20·0 ± 1·7	85 ± 6	365 ± 22	153 ± 11	16·2 ± 3·6

* t test of difference in pH. 1:2, N.S.; 1:3, $P < 0.001$; 3:4, $P < 0.001$.
† Data obtained at the beginning of the experimental period.
‡ Data obtained two days after returning the toads from 500 mOsm NaCl back to tap water.

Table 3. *The effect of amiloride* (5×10^{-4} M) *in tap water on blood data analyses in the toad* Bufo viridis

(Average weight of the animals was 55 ± 5 g, and that of the haematocrit 16 ± 2 %; t = 23 °C (1979). After control determination (with the toads in 3 cm deep tap water), the toads spent 3–4 days in amiloride-containing tap water. Mean ± S.E.M. Number of toads in parentheses.)

	pH	P_{CO_2} (mmHg)	HCO_3^- (mM)	Osmolarity (mOsm)	Cl^- (mM)	Haematocrit (%)
1. Control (9) (tap water)	7·745 ± 0·027	11·1 ± 0·6	19·3 ± 1·7	340 ± 15	136 ± 6	17 ± 2
2. 3 days in amiloride (10)	7·528 ± 0·027	9·2 ± 0·4	9·5 ± 0·6	281 ± 9	125 ± 8	17 ± 3
3. Control group* (5)	7·751 ± 0·045	10·8 ± 0·3	19·6 ± 2·6	341 ± 17	133 ± 8	17 ± 3

* A group of toads which were used for analyses in parallel to the experimental group, but in tap water.

Table 2. *The effect of amiloride and salt adaptation on the* in vitro *Na^+/H^+ exchange across the isolated skin of the toad* Bufo viridis

(Method according to Ehrenfeld & Garcia Romeu (1977).* Ringer–bicarbonate on the inside and buffered 2 mM NaCl on the outside of the skin. A, skins from toads previously adapted in deionized water. B, skins from toads previously adapted in 600 mOsm NaCl for at least 10 days. Mean ± S.E.M. Number of separate skins in parentheses.)

	Na^+ net flux (nequiv. $cm^{-2}.h^{-1}$)	H^+ net flux	Potential difference (mV)
	A (deionized water)		
1 Control (9)	140 ± 11	−160 ± 14	70 ± 1
2 Amiloride (10^{-4} M) (5)	−68 ± 26	−12 ± 16	2 ± 1
	B (600 mOsm NaCl)		
1 Control (6)	−10 ± 18	−25 ± 8	−20 ± 3
2 Amiloride (10^{-4} M) (4)	−111 ± 85	40 ± 21	−23 ± 3

* Experiments done in collaboration with Drs F. Garcia-Romeu and J. Ehrenfeld in Nice.

water (Table 2A). The potential difference across the skin and the secretion of protons disappeared, while the net flux of sodium reversed. The reversal in the net flux is the result of increased efflux, with 120 mM sodium on the inside and only 2 mM on the outside, which is favoured when the entry pathway for sodium is blocked by amiloride (Biber & Mullen, 1977).

The net fluxes across the skin of animals adapted for 10 days in 600 mOsm NaCl are completely abolished (Table 2, B). The effect of amiloride on these skins was seen mainly on the efflux of sodium, which increased by an order of magnitude. It shows that despite the great reduction in the amiloride-sensitive sodium transport across the skin (i.e. entry sites; Katz, 1975), the recycling mechanism (Hviid-Larsen, personal communication, 1980) is operating rather effectively. These experiments (Table 2, A, B) were carried out under similar experimental conditions (the osmolarity and the composition of the bathing solutions) and may therefore be compared. This comparison demonstrates a similarity in the net fluxes of sodium and hydrogen ions between the skins from deionized water-adapted toads treated with amiloride, and the skins from the high-salt-adapted toads, under control conditions. Since the effect of amiloride on the net fluxes of sodium and hydrogen *in vitro* was similar to the effect of salt adaptation, it was decided to determine the effect of this drug on the acid–base status of the blood *in vivo*. Table 3 summarizes the results of experiments in which toads were immersed for 2–4 days in tap water containing 5×10^{-4} M amiloride. It can be seen that there is a marked reduction in the pH and the concentration of HCO_3^-, while the P_{CO_2} of the blood did not change. These effects are characteristic of metabolic acidosis. The osmolarity of the blood even decreased a little. Comparison of Tables 1 and 3 shows that the acid–base status of the blood *in vivo* is affected similarly by the two experimental conditions (e.g. salt adaptation and amiloride).

DISCUSSION

The present study gives an account of the acid–base status and the blood gases of the toad *in vivo* under conditions of adaptation to high salinity. A great deal of

the CO_2 elimination in amphibia is carried out through the skin (Krogh, 190[illegible] Rahn & Howell, 1976) and is characteristic of the bimodal gas exchange of this group (Lenfant, Johansen & Hanson, 1970). The skin of amphibia is therefore of prime interest, being endowed with both respiratory exchange mechanisms, and with solutes and water-exchange mechanisms (Krogh, 1904, 1934; Garcia-Romeu, 1971; Ziegler *et al.* 1974).

Salt adaptation of the euryhaline toad *B. viridis* (Katz, 1973) is accompanied by marked physiological changes. The results reported in this study show that the blood gases (i.e. P_{O_2} and P_{CO_2}) in the toad are not affected either by adaptation in high salt solution for over 6 days (they can live under these conditions for over a month; Katz, 1973), or in tap water containing amiloride (Tables 1 and 3). These conditions do not, therefore, affect the ability of the respiratory and circulatory systems to sustain their function in maintaining normal levels of the blood gases. In fact Jackson & Braun (1979) have shown recently that the regulation of blood P_{CO_2} in the bullfrog is carried out mostly, if not solely, by the pulmonary system.

The pattern of the changes in the acid–base status of the blood under salt adaptation (Table 1) is characteristic of metabolic acidosis (Pitts, 1974), and is reflected in a marked acidification of the blood and a simultaneous reduction in the concentration of bicarbonate. In the relevant reaction:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-, \quad (1)$$

CO_2, which is continuously produced by the tissues, would drive it to the right. The analysis of the regulatory mechanisms responsible for the acid–base regulation under our experimental conditions is rather complex. In the amphibia it involves at least the kidneys, the urinary bladder and the skin, and may also include the respiratory system (Jackson & Braun, 1979). Although the kidneys may play an important role in the regulation of the acid–base status, the study of Yoshimura *et al.* (1961) in the bullfrog pointed to the limited capacity of the renal mechanism in the frog to cope with extreme acidosis. A striking similarity was observed in this study in the *in vivo* acid–base status of the blood under salt adaptation (Table 1) as compared to that which was found in toads kept in tap water containing amiloride (Table 3); the latter condition inhibits the uptake of sodium across the skin (Kirschner, Greenwald & Kerstetter, 1973). This was correlated with a similarity in the Na^+/H^+ exchange across the isolated skin determined *in vitro* in the two experimental conditions (Table 2, A, B). Metabolic acidosis is induced then, both in salt adaptation and by amiloride, while blood osmolarity is greatly increased only under high salt adaptation. Since both conditions block sodium transport across the toad skin (Katz, 1975), which results in a diminution of hydrogen secretion, it suggests that the skin may play an important role in the control of acid–base status in toads. A similar conclusion has been reached by Vanatta & Frazier (1980), who studied *Rana pipiens* skins *in vitro* after *in vivo*-induced alkalosis and acidosis.

In fish, Renzis & Maetz (1973), and Cameron (1978), have postulated an analogous role to the gills in regulating the acid–base status of the blood.

In conclusion, the present study has demonstrated the effect of salt adaptati[illegible]

the *in vivo* acid–base status of the blood in the toad. The metabolic acidosis was related to an inhibition of hydrogen secretion through the skin, as a result of the inhibition of sodium transport under these conditions. A quantitative assessment of the relative contribution of the various organs (i.e. the kidneys, the urinary bladder and the skin), to the regulation of acid–base status under adaptation to high salinities will throw more light on the regulatory mechanisms involved.

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