A new function for lactate in the toad *Bufo marinus*

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Pörtner, H. O., L. G. S. Branco, G. M. Malvin, and S. C. Wood. A new function for lactate in the toad *Bufo marinus*. *J. Appl. Physiol.* 76(6): 2405–2410, 1994.—In the amphibian *Bufo marinus*, progressive hypoxia below a critical P_{O_2} elicits a transient 50% increase in O_2 consumption that coincides with the onset of lactate formation. The present study was designed to test the hypothesis that lactate causes the observed rise in metabolic rate. Arterial bolus infusions of pH-neutral sodium lactate solutions (4 mmol/kg body wt) in toads maintained under hypoxia actually elicit a similar increase in metabolic rate. The application of adrenergic antagonists (bretylium tosylate, phentolamine, propranolol, and reserpine) inhibits this response, suggesting that catecholamines are involved. Moreover, animals injected with lactate move to a cooler environment (behavioral hypothermia), a behavioral response that is beneficial during hypoxia. We hypothesize that, in accordance with Cannon's concept of an emergency response, lactate may function as an alarm signal during hypoxia. However, the signal function of lactate is observed in animals both under hypoxia and under normoxia and should thus be considered in future studies whenever elevated lactate levels are present, e.g., during and after exercise.

behavior; emergency response; exercise; hypothermia; hypoxia; metabolic rate; oxygen consumption; oxygen debt

HYPOXIA is a common environmental condition for fossorial or diving animals. Many amphibians are prone to hypoxia, because they spend considerable time underground or under water as protection from heat or desiccation. Tolerance to hypoxia can be quantified by an analysis of the critical P_{O_2} (Pc), which is the threshold P_{O_2} for changes in various physiological parameters and, most importantly, for the onset of anaerobic energy production (15). During progressive hypoxia below Pc, the molar rate of O_2 consumption ($\dot{M}O_2$) changes. In many species it falls, indicating the failure to maintain a constant regulated metabolic rate (e.g., see Ref. 20). This finding, however, is not consistent among all species. In some lower vertebrates (e.g., goldfish or rainbow trout) a transient increase in $\dot{M}O_2$ was recorded (although sometimes overlooked) before it finally fell (2, 21). In the toad *Bufo marinus*, a steady state close to a 50% increase in metabolic rate was monitored. This increase occurred at hypoxia slightly below Pc when lactate rose in the plasma above a threshold level of 0.5–1.0 mmol lactate/l (16, 29). Other changes associated with Pc in amphibians are an increase in hematocrit, a drop in body CO_2 stores, changes in the acid-base status, and the selection of a cooler environment (i.e., behavioral hypothermia; Refs. 9, 16, 17, 29). This behavioral reaction increases hypoxia tolerance, since it will cause a fall in body temperature and, accordingly, a decrease in metabolic rate. It will allow a larger fraction of metabolism to remain aerobic, as indicated by a correlated shift of Pc to lower values (3).

How do animals sense severe hypoxia and, especially, the transition to anaerobic metabolism? On the basis of a correlated increase in $\dot{M}O_2$ and in plasma lactate levels during a period of respiratory alkalosis in *Bufo marinus*, we hypothesized that the lactate anion elicits the increase in metabolic rate (16, 17). This hypothesis suggested a function for lactate as a signal that changes the metabolic status of the animal as it is formed. The present study was designed to test this hypothesis and to evaluate whether the appearance of lactate in the plasma causes the behavioral hypothermia. We also tested whether catecholamines contribute to these responses as hypothesized by Pörtner et al. (16). To exclude a stimulating effect of H^+ on catecholamine release that has been established in hypoxic lactacidotic amphibians (19), we used sodium lactate (NaLa) infusions to investigate the specific function of the lactate anion.

MATERIALS AND METHODS

Animals and experimental procedure. Maintenance and treatment of the experimental animals [adult *Bufo marinus* toads, 226 ± 11 (SD) g body wt] before experimentation was described previously (16, 29). For $\dot{M}O_2$ measurements, the ischiadic artery of one hind leg was cannulated and the animals were placed in darkened chambers (volume 2.0–2.2 liters) containing 200 ml of dechlorinated tap water. Temperature was maintained at 20 ± 0.5°C.

$\dot{M}O_2$ was measured using Ametek Applied Electrochemistry (Pittsburgh, PA) S3A and S3AII O_2 analyzers. Gases were prepared from pure O_2 and N_2 with a Corning (Medfield, MA) gas mixer, saturated with water at 20°C, and then fed into the animal chambers at a flow rate of 55.6 ml/min. Readings were taken from inlet and outlet gas mixtures after drying and removal of CO_2 (25). Rapid changes in $\dot{M}O_2$ were evaluated considering the washout characteristics of the system using the equations provided by Bartholomew et al. (1).

The hypothesis that the lactate anion elicits an increase in metabolic rate during hypoxia was tested under low P_{O_2} (7% O_2, 5.6 kPa, 42 Torr at Albuquerque altitude of 1,600 m). This O_2 level was chosen because it is slightly above Pc (4.0–4.7 kPa, 30–35 Torr at 20°C), thereby excluding endogenous lactate formation (16). Because lactate is a substrate of aerobic metabolism (see Fig. 1*B*), a hyperosmotic solution containing a high concentration of NaLa was chosen for a bolus infusion (0.8 mol/l, 5 ml/kg body wt = 4 mmol/kg) to extend the period when lactate was present and to allow for long-term recordings of a potential lactate effect. $\dot{M}O_2$ was measured during a stepwise reduction in P_{O_2} (from 20 to 12 to 7% O_2) before an arterial sham injection of NaCl and the subsequent infusion of an identical volume of NaLa. After complete degradation of the infused lactate and subsequent return to normoxia (20% O_2, 17.3 kPa, 130 Torr), animals were reinjected with lactate to test whether the effect of lactate as a metabolic stimulus depends on the ambient O_2 level.

Blood samples (~0.4 ml) were withdrawn from the undisturbed animal via the indwelling catheter. Arterial pH was measured using a thermostatted Radiometer (Copenhagen, Denmark) BMS 3 system. Plasma was obtained by centrifuga-

tion and was used for the analysis of lactate after perchloric acid extraction and neutralization (Ref. 16; see Figs. 1 and 2).

To eliminate adrenergic responses, animals were fed reserpine tablets (2.5–3.0 mg/kg; to deplete catecholamine stores) and were injected with 10 mg/kg of bretylium tosylate once daily on two consecutive days (to deplete adrenergic nerve endings) before the day of the experiment. Phentolamine mesylate (2 mg/kg; α-adrenergic blockade), propranolol hydrochloride (2 mg/kg; β-adrenergic blockade), and bretylium tosylate (10 mg/kg) were also injected ~1 h before lactate was infused. A mixture of the different substances was chosen for a maximum effect and to reduce the risk of side effects by the application of high levels of individual agents (e.g., reserpine and phentolamine also inhibit the 5-hydroxytryptamine system, and propranolol has a nonspecific anesthetic action on tissues). All drugs were dissolved in saline before injection via the arterial cannula. $\dot{M}O_2$ of the pretreated animals remained constant after phentolamine, propranolol, and bretylium tosylate infusions and was not significantly different from the untreated control animals [2.79 ± 0.29 (SE) vs. 2.62 ± 0.20 mmol · $kg^{-1} \cdot h^{-1}$, respectively; $n = 6$]. Propranolol and phentolamine were readministered 2 h after lactate infusion, since their effects decrease over time (23). There was no indication that the ability of the animals to move about was depressed during or after experimentation.

Behavioral thermoregulation of toads (299 ± 15 g) was studied in a temperature gradient in a sealed darkened 150-liter chamber (1.1 m long; Ref. 29). One end was heated to ~40–45°C by a heated plate. The other end was cooled to ~10°C by having chilled polyethylene glycol beneath the floor. Petri dishes filled with water were placed along the temperature gradient providing free access to water at all temperatures. Each animal was fitted with an ischiadic arterial cannula and two thermistors (Yellow Springs Instruments, Yellow Springs, OH) secured to the animal by suturing to the skin; one was inserted ~2 cm in the cloaca and the other was attached to the belly in the middle of the pelvic patch. Rapid temperature changes recorded from the latter confirmed that the animal moved to different temperatures. The toad was placed in the middle of the temperature gradient; the chamber was provided with a mixture of 44% N_2 in air (P_{O_2} ~70 Torr, allowing an elevated Pc at higher temperatures) at ~760 ml/min using a Wösthoff 301 a/F (Bochum, Germany) gas mixing pump. After an initial control period, when the animal had moved to its preferred temperature, the effects of NaCl and NaLa infusions were tested. Because infusion of a hyperosmotic NaCl solution caused hypothermia by itself (see Fig. 3*A*), a similar protocol was repeated with isosmotic solutions (0.11 mol/l, 10 ml/kg; see Fig. 3*B*).

Changes over time were tested for significance at the 5% level by using one-way (Figs. 1 and 3) or two-way (Fig. 2) repeated measures analysis of variance and by performing contrasts for group comparisons and for comparisons of different treatments.

RESULTS

The increase in $\dot{M}O_2$ caused by the initial injection of a hyperosmotic NaCl solution under 7% O_2 was small, nonsignificant, and rapidly reversed (Fig. 1). Subsequent injection of the same quantity of NaLa led to a large increase in $\dot{M}O_2$ that also fell rapidly during the 1st h. After 1 h, however, $\dot{M}O_2$ decreased slowly from values that were 36% higher than hypoxic control values down to values 20% above control values after 12 h. During this time period, plasma lactate levels fell to 1.5 mmol/l. $\dot{M}O_2$ and plasma lactate levels fell in a correlated fashion thereafter. Reinfusion of lactate after return to 20% O_2

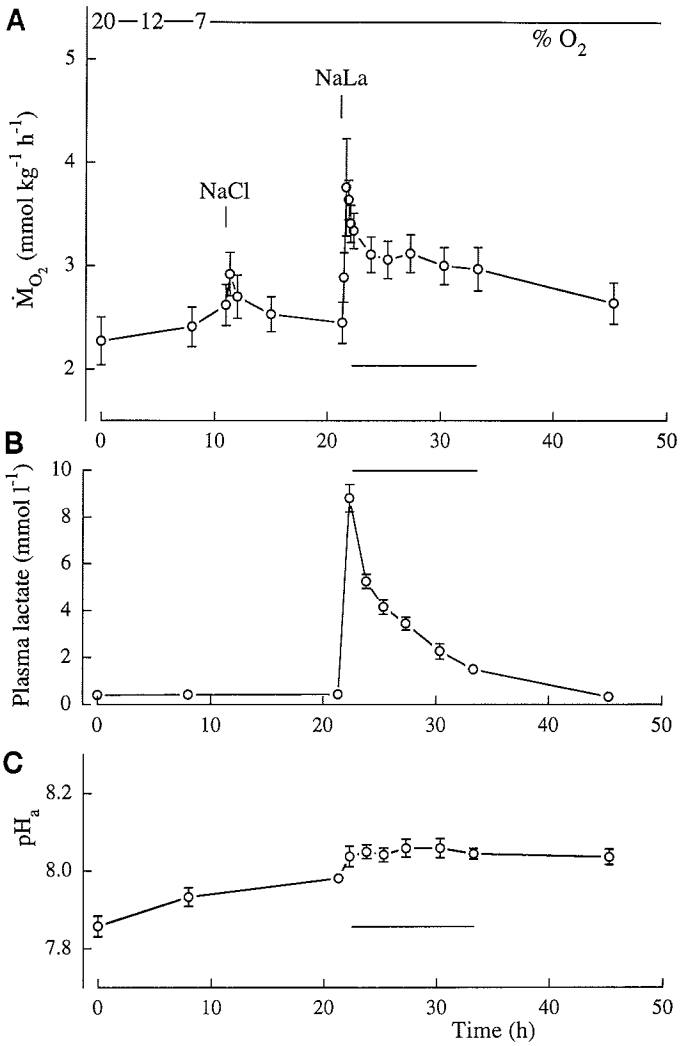

FIG. 1. Changes in molar rate of O_2 consumption ($\dot{M}O_2$; *A*), plasma lactate levels (*B*), and arterial pH (pH_a; *C*) with time during stepwise reduction in P_{O_2} before arterial sham injection of NaCl and subsequent infusion of identical volume of sodium lactate (NaLa). Values are means ± SE; $n = 6$. Injection of NaCl excluded that potential increase in salt or ion concentrations linked to NaLa injection could have large and persistent influence on metabolic rate by itself. Horizontal lines, onset and maintenance of significant effect of previous treatment.

demonstrated that the rise in $\dot{M}O_2$ was even higher (by 55% from 2.4 ± 0.3 to 3.7 ± 0.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ after 2 h) and was thus not restricted to low P_{O_2}.

Measurements of arterial pH before and after lactate infusions confirmed that the injection of the sodium salt of lactic acid excluded the development of an acidosis, thus emphasizing that lactate anions alone cause the long-term increase in metabolic rate (Fig. 1*C*). A slight alkalosis developed instead, in addition to the respiratory alkalosis seen previously under the same conditions (17). Such a rise in pH is in accordance with a metabolic alkalosis expected from lactate catabolism. The additional alkalosis was more or less reversed with the disappearance of lactate from the plasma.

The application of a cocktail of adrenergic antagonists significantly reduced the increase in $\dot{M}O_2$ (Fig. 2*A*), leading to lower rates of lactate removal from the plasma (Fig. 2*B*) with similar pH changes (Fig. 2*C*). As stated

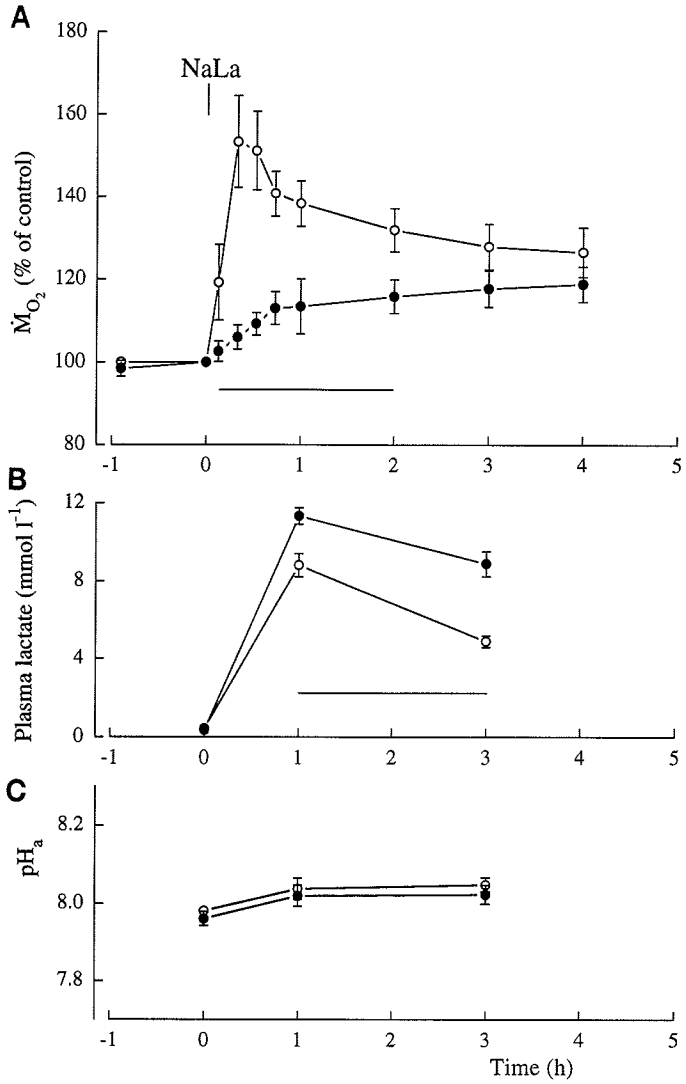

FIG. 2. Changes in $\dot{M}O_2$ (*A*), plasma lactate levels (*B*), and pH_a (*C*) in untreated animals (○; animals under 7% O_2; see Fig. 1) and after application of α- and β-adrenergic antagonists (●) before infusion of lactate. Values are means ± SE; $n = 6$. Increase in $\dot{M}O_2$ was significantly reduced for 2 h and was linked to plasma lactate levels significantly higher than control levels, indicating a reduced rate of catabolism of infused lactate. Horizontal lines, onset and maintenance of significant difference between untreated animals and specimens treated with antagonists.

above, the applied cocktail can affect other systems. Consequently, the effects of the cocktail may not be the effect of adrenergic inhibition alone. However, the results are consistent with catecholamine mediation and support the conclusion that catecholamines are involved in the chain of events leading from an increase in lactate levels to the observed increase in metabolic rate. Moreover, parallel investigations demonstrated that a large rise in heart rate observed after lactate infusions in untreated control animals was not observed in animals treated with adrenergic antagonists.

To test the hypothesis that the lactate signal causes behavioral hypothermia, we first applied a protocol similar to the one used for $\dot{M}O_2$ analyses (Fig. 1). Hypoxic fully hydrated toads injected with a hyperosmotic solution of NaCl already selected a mean temperature slightly (2.1 ± 1.4°C) but significantly below the control value of 28.2 ± 1.3°C (Fig. 3*A*). Subsequent infusion of the hyperosmotic NaLa solution caused an additional slightly larger drop in the preferred body temperature that was significantly below both control values (by 4.5 ± 1.5°C; $P < 0.01$) and the temperature preferred after NaCl injection (by 2.6 ± 0.5°C; $P < 0.01$). A stimulatory effect of hyperosmotic NaCl solutions alone would be in line with behavioral hypothermia observed in toads during dehydration when plasma Na^+ or Cl^- levels may be elevated (10). However, the effect of NaCl could be eliminated and a specific lactate effect could be demonstrated when isosmotic solutions were used, leading to a temperature drop of 2.9 ± 1.0°C only when lactate was injected ($P < 0.01$, Fig. 3*B*).

DISCUSSION

Metabolic stimulation. In accordance with our previous hypothesis, our results confirm that the lactate anion alone is able to cause an increase in metabolic rate in

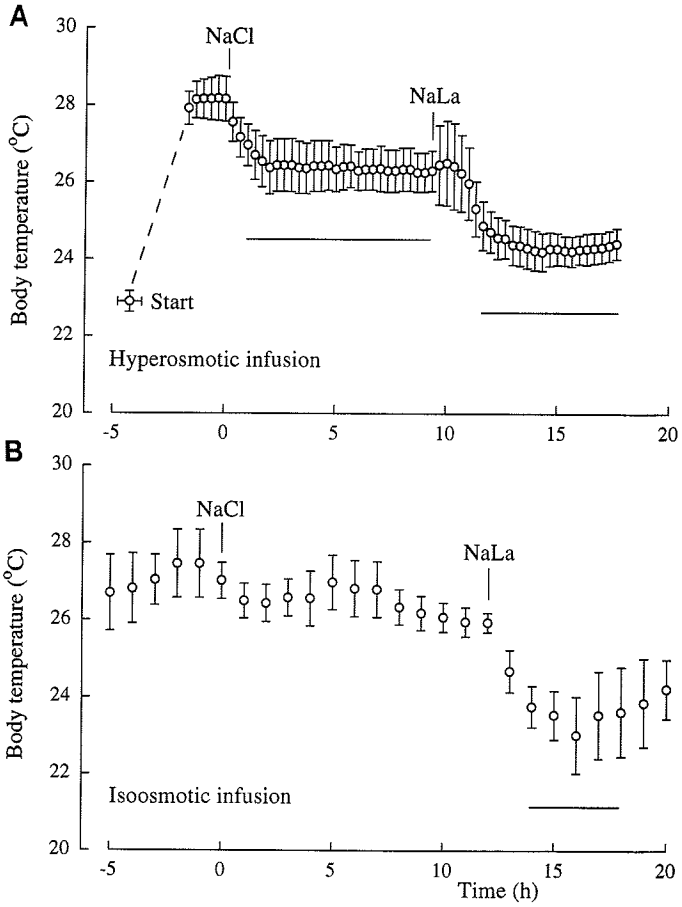

FIG. 3. Preferred body temperature under control conditions, after arterial sham injection of NaCl, and after consecutive infusion of NaLa. Values are means ± SE; $n = 5$. Temperature changes were caused by locomotion of animals in temperature gradient. *A*: effect of hyperosmotic solution. *B*: effect of isosmotic solution. Because infusion of hyperosmotic NaCl solution caused hypothermia by itself (*A*; see text), a similar protocol was repeated with isosmotic solutions after extended 12-h control period (not fully shown), indicating that behavioral hypothermia was specifically elicited by elevated plasma lactate levels (*B*). Horizontal lines, onset and maintenance of significant effect of previous treatment.

hypoxic animals. Moreover, lactate is still effective in animals under normoxia. The difference in the responses to NaCl and NaLa also supports our conclusion that lactate (and not sodium or the potential increase in salt or ion concentrations) causes a large and persistent increase in metabolic rate (Fig. 1). After the infusion of lactate, $\dot{M}O_2$ only fell to control values when plasma lactate levels decreased below 0.5–1.0 mmol/l, suggesting that the magnitude of the response is not dependent on the actual lactate concentrations in the plasma. These observations are consistent with our earlier conclusion that lactate becomes effective as a metabolic stimulant above a certain plasma threshold level (16). After lactate infusions during normoxia, the larger increase in $\dot{M}O_2$ may be related to the increased aerobic scope of metabolism at higher O_2 levels. In support of our finding of a stimulating effect of lactate on the metabolic rate, a close temporal correlation between elevated lactate and $\dot{M}O_2$ levels was previously described for alligators after exercise and also after NaLa injections (Ref. 4; for a discussion of the role of lactate during exercise, see below). A similar trend (although not reported to be significant under the applied experimental conditions) was observed after lactic acid and NaLa infusions in a varanid lizard (12).

It might be argued that the process of lactate removal is reponsible for the increase in metabolic rate. In anuran amphibians, both oxidative catabolism and, even more so, the use of lactate in anabolic muscle glycogenesis are the predominant mechanisms of lactate removal (6, 26). Any use of lactate in oxidative catabolism would follow the energy requirements of the organism and would not be able to explain an increase in metabolic rate by itself (the use of other substrates would just be reduced). This conclusion is supported by the suggested coupling between the action of lactate and the effect of catecholamines.

Therefore, only an increase in anabolism, i.e., net glycogen synthesis from injected lactate, could cause an actual increase in ATP requirements. For a maximum estimate of the required energy, it may hypothetically be assumed that all injected lactate is converted into glycogen. The excess $\dot{M}O_2$ observed during 24 h after lactate infusion (see Fig. 1) equals 40 mmol ATP/kg body wt (using a P/O ratio of 3). This amount far exceeds the maximum ATP quantity (16 mmol ATP/kg) required for glycogen synthesis from lactate. Obviously, it is not lactate as a metabolic substrate that elicits the increase in $\dot{M}O_2$, but its major effect is likely to be mediated via catecholamines. In support of this conclusion, glycogen synthesis would even be inhibited by the effect of epinephrine.

In this context, it must be reemphasized that lactate was metabolized in our experiments, since a P_{O_2} had to be chosen that allowed us to demonstrate the specific effect of the lactate anion on infusion into the hypoxic but completely aerobic animal. This was not the case in our previous study when the increase in metabolic rate occurred in hypoxic untreated toads below Pc as soon as lactate was formed and not when it was metabolized (16). Net glycogen is depleted under these conditions, and therefore the rise in metabolic rate cannot be explained by an increase in anabolic ATP requirements. Therefore, only in fully aerobic animals can the lactate effect mediated by catecholamines cause a correlation between lactate depletion and metabolic rate increase in time. As outlined above, this must not necessarily include a correlation in quantity. In some species the reported temporal correlation may even be eliminated by a reduction of the adrenergic response during long-term exposure to lactate (e.g., in hypoxia-acclimated animals; see above).

Although toads become restless below Pc (see below), an increase in activity level would very likely cause fluctuating rates of $\dot{M}O_2$ and, therefore, is unlikely to explain the maintenance of a long-term steady-state elevation in $\dot{M}O_2$ slightly below Pc (for 24 h at P_{O_2} = 21 Torr; Ref. 16) or after lactate infusion (see Fig. 1). An increased cost of ventilation is also unlikely to be a major factor, since this cost is low in amphibians (5%; Ref. 11) and would not cause a sharp 50% rise in $\dot{M}O_2$ below Pc. It would rather be expected to increase steadily with progressive hypoxia. Moreover, the response reported here may be eliminated during long-term hypoxia acclimation (18) when Pc may be shifted to lower values (15) and the aerobic scope of metabolism decreases.

After lactate infusions under both hypoxia and normoxia, an increase in ventilation would also be unlikely to explain the observed sudden rise in $\dot{M}O_2$ (which evidently also happens when there is no limitation in O_2 supply). The contribution of catecholamines suggests that a calorigenic increase in metabolic rate (i.e., a rise in heat production because of the futile cycling of metabolic substrates and products) occurs as a response to the presence of lactate. Such an increase in metabolic rate is a common correlate of an emergency response and could prepare the organism for a "fight-or-flight" reaction.

Behavioral hypothermia. An emergency response would also imply an increased awareness of the animals and an adequate behavioral reaction. A previous study demonstrated that the animals become restless and seek a cooler temperature below Pc (29). This behavior is beneficial, since it increases hypoxia resistance. The accumulation of lactate during hypoxia very likely causes behavioral hypothermia by stimulating the animals to move to cooler temperatures in a temperature gradient (Fig. 3*B*).

The temperature drop elicited by arterial lactate infusions was smaller than previously observed during transition to hypoxia below Pc (by 8.5°C; Ref. 29). The response of *Bufo marinus* to its major anaerobic end product is similar to the behavioral hypothermia reported for goldfish exposed to or injected with its anaerobic end product, ethanol (5, 13). Crawshaw et al. (5) demonstrated that only intracranial ethanol infusion elicited a maximum drop in the preferred temperature close to the one previously seen in *Bufo marinus*. The authors assumed a link between the presence of ethanol and the release of norepinephrine that also causes behavioral hypothermia (27). Similarly, lactate may cause a central response in hypoxic *Bufo marinus*, including the release of catecholamines that elicit both the observed increase

in metabolic rate and the behavioral hypothermia. Lactate infused into the systemic circulation of fully aerobic animals would have to cross the blood-brain barrier to become effective and would also be rapidly metabolized. It may very well be that arterial lactate infusions in *Bufo marinus* did not bring intracranial lactate up to those levels required for the maximum hypothermia seen during transition to severe hypoxia.

Perspectives and conclusions. The increase in metabolic rate observed below Pc is quite unexpected compared with the economic drop in energy turnover found in ectothermic vertebrate anaerobes during long-term exposure to severe hypoxia far below Pc (7, 8, 22). We report here an increase in metabolic rate that begins as soon as lactate appears in the plasma but is most likely transient during more severely hypoxic exposure when metabolism falls below the standard metabolic rate (15). The mechanisms leading from an initial increase in metabolic rate to the passive long-term toleration of anaerobiosis during progressive hypoxia require further investigation. The questions of which tissues are involved in the futile cycling of substrates and whether the awareness response also involves a transient rise in anaerobic metabolism below Pc also arise.

Our experiments demonstrate that the signal function of lactate as such is independent of the ambient Po_2. As a corollary, a signal function of lactate mediated by catecholamines should be considered whenever lactate is formed. This mechanism would very likely support metabolic adjustments during exercise and would maintain high metabolic rates during recovery from exercise. Actually, the mechanism discussed in our study might also explain why the repayment of an O_2 debt and the O_2 quantity required for the restoration of energy stores does not balance in many species (e.g., in rainbow trout; Ref. 28). Future studies must show whether other hormones are also involved or may synergistically support the observed reaction.

Generally, the signal function of lactate may represent a major evolutionary advantage of lactic acid formation over the use of other glycolytic end products like the opines that are found in marine invertebrate groups. Opines are not released into the blood but are recycled at the site of production (14, 24). As a prerequisite for a signal function of lactate, pH-dependent distribution of lactic acid between body compartments allows for a rapid release and the predominant accumulation of lactate in the plasma that is frequently observed during hypoxia (14).

In summary, the change in metabolic rate linked to an increased awareness of hypoxic animals and their behavioral adjustment demonstrate that the onset of lactate formation and its accumulation in *Bufo marinus* represent a signal to the organism. Lactate is, therefore, not only an anaerobic metabolic end product but it both signals to the animal its unfavorable situation and brings it to a higher metabolic level and causes it to move to a more suitable environment. In accordance with Cannon's concept of an emergency response, lactate could thus be considered an alarm signal that is a metabolic mediator between environmental changes (progressive hypoxia) and the appropriate behavioral reaction of the animal (behavioral hypothermia). From a general point of view, the lactate effect on metabolic rate and behavior should also be considered in future studies of exercise metabolism, O_2 debt analysis after exercise, thermoregulation, and, possibly, stress physiology. It remains to be established whether the proposed functions of lactate also occur in endothermic animals (mammals and birds) and among lactate-forming invertebrates.

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