8-6-1982

Aspects of Anuran Metabolism: Effects of Chronic Hypoxia on Maximal Oxygen Uptake Rates and the Fate of Lactic Acid

Thomas Charles Solberg
Portland State University

AN ABSTRACT OF THE THESIS OF Thomas Charles Solberg for the Master of Science in Biology presented August 6, 1982.

Title: Aspects of Anuran Metabolism: Effects of Chronic Hypoxia on Maximal Oxygen Uptake Rates and the Fate of Lactic Acid

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

[redacted]

Stanley S. Hillman, Chairman

[redacted]

Philip C. Withers

[redacted]

Richard B. Forbes

Some aspects of anuran metabolism are examined, with special emphasis on possible limitations to aerobic metabolism and the effect of chronic hypoxia acclimation on maximal rates of aerobic metabolism and the metabolic fate of lactic acid accumulated after anaerobic metabolism.

During exposure to hypoxia, oxygen delivery could possibly impose a limit on maximal rates of oxygen consumption ($\dot{V}O_2$,max). The $\dot{V}O_2$,max in *Xenopus laevis* did not decrease with a decline in ambient P_{O_2} to 100 torr. At P_{O_2} less than 100 torr, $\dot{V}O_2$,max declined and

was highly correlated with ambient P_{O_2}.

Chronic hypoxia acclimation increased the blood oxygen capacity of *Xenopus laevis*, due mainly to polycythemia. There was no increase in $\dot{V}_{O_2}$,max after hypoxic acclimation except at the lowest P_{O_2} tested (P_{O_2} less than 38 torr).

The fate of lactic acid accumulated after activity was examined in *Bufo americanus*, *Rana pipiens* and *Xenopus laevis* using C^{14}-labelled lactic acid. Less than 5% of the C^{14} activity appeared as expired $C^{14}O_2$ in all animals. *Rana pipiens* accumulated large amounts of the C^{14} activity in the muscles. C^{14} activity in *Bufo americanus* was more evenly distributed throughout many tissues, with the highest concentrations in the blood, liver, lungs and ventricle.

ASPECTS OF ANURAN METABOLISM:

EFFECTS OF CHRONIC HYPOXIA ON MAXIMAL OXYGEN UPTAKE RATES AND THE FATE OF LACTIC ACID

by

THOMAS CHARLES SOLBERG

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University

1982

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of Thomas Charles Solberg presented August 6, 1982.

[redacted]

Stanley S. Hillman, Chairman

[redacted]

Philip C. Withers

[redacted]

Richard B. Forbes

APPROVED:

[redacted]

W. Herman Taylor, Head, Department of Biology

[redacted]

Stanley E. Rauch, Dean of Graduate Studies and Research

ACKNOWLEDGEMENTS

A special debt of gratitude is owed Drs. Stan Hillman and Phil Withers. Their advice, support and patience made this thesis possible, if not inevitable. Their friendship and guidance will not soon be forgotten.

My colleagues, the biology graduate students of Portland State, especially Scott Landrey, James Campbell and Nancy Broshot (the old timers) made this experience much more rewarding and enjoyable.

My parents made it all possible. Their support in all of my endeavors, especially the ones they didn't fully understand, was unwavering and greatly appreciated.

Special thanks to my wife, Mitch, who made it all bearable and worthwhile. When no light was at the end of the tunnel, she lead the way.

May we all meet at the Fog on Friday.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Ectothermic vertebrates rely to a large extent upon anaerobic metabolism during high levels of activity (Bennett and Licht, 1973; Hutchison and Turney, 1975; Bennett, 1978). The extent of the anaerobic contribution to activity metabolism has been extensively studied (Bennett and Licht, 1973; Hutchison et al., 1977; Hutchison and Miller, 1979b) but these experiments used electrical shock to induce activity, and manometric techniques for the measurement of oxygen consumption. Manometric methods seriously underestimate oxygen consumption due to electrolytic gas generation and temperature transients in the manometer chamber (Hillman et al., 1979). Electrical stimulation may also cause excessive production of muscle lactic acid and decreased blood lactic acid levels during activity due to circulatory impairment (Putnam, 1979; Hillman et al., 1979).

Glycogen, the polymeric storage form of glucose, is catabolized to pyruvate by the metabolic process known as glycolysis. Pyruvate is then the substrate for oxidation to carbon dioxide and water by the tricarboxylic acid cycle and the mitochondrial electron transport system. This complete oxidation of glucose yields a net 36 ATP per molecule of glucose (Lehninger, 1975). When oxygen is unavailable, the pyruvate formed from glycolysis may be catalysed by lactate dehydrogenase to form lactic acid, with a net yield of two ATP per glucose molecule. This conversion step from pyruvate to lactate is

important because it regenerates NAD^+ from NADH formed in an earlier step in glycolysis (see Figure 1).

There are both advantages and disadvantages to aerobic and anaerobic metabolism. Anaerobic metabolism is much faster than aerobic metabolism since oxygen is not needed by the tissue. Consequently, there is no time lag involved in increasing oxygen transport (i.e. ventilation and the circulation of blood). Anaerobic metabolism is, therefore, very important in burst activity such as escape from predation (Bennett and Licht, 1974). Anaerobic metabolism is less dependent on temperature than aerobic metabolism (Bennett, 1978; Carey, 1979a; Hochachka, 1980; Putnam and Bennett, 1981). This is especially important for ectotherms such as amphibians. The net yield of only two ATP per glucose molecule by anaerobic metabolism, as compared to 36 ATP produced aerobically indicates a relative inefficiency of anaerobic metabolism. Other limitations include the accumulation of potentially toxic lactic acid and the associated pH effects (metabolic acidosis), and the requirement of anaerobiosis of glucose as its substrate; aerobic metabolism can utilize lipids and proteins as well as glucose.

Anuran amphibians provide an excellent model system for the study of anaerobic and aerobic metabolism due to the considerable interspecific differences in methods of energy generation. *Rana pipiens* and *Rana catesbeiana*, for example, rely mainly an anaerobic metabolism during bursts of activity; they have a low maximal rate of oxygen consumption ($\dot{V}_{O_2}$,max) and high lactate concentrations during activity (Bennett and Licht, 1974; Hutchison and Turney, 1975;

Hillman, 1976; Hillman et al., 1979; Hillman and Withers, 1979; Hutchison and Miller, 1979a; Putnam, 1979a). Other anurans such as *Bufo cognatus* and *Bufo boreas* have a much higher $\dot{V}_{O_2}$,max and lower lactate concentrations during activity (Bennett and Licht, 1974; Hillman, 1976; Hillman and Withers, 1979; Carey, 1979b; Putnam, 1979a).

It has been hypothesized that this apparent dichotomy in metabolic "strategies" is associated with predator avoidance mechanisms (Bennett and Licht, 1974). The more-anaerobic anurans depend on rapid, usually saltatory, movements to escape from predators, whereas the more-aerobic species use static defense mechanisms such as lung inflation or skin poisons. There are, however, many species that do not readily fit into either the predominantly aerobic or anaerobic modes. *Xenopus laevis* is an example of an anuran with both a high $\dot{V}_{O_2}$,max and high lactate concentrations during activity (Hillman, 1976; Hillman and Withers, 1979; Putnam, 1979b).

Taigen et al. (1982), in a survey of a wide variety or anurans, have found a continuum of metabolic strategies rather than a dichotomy. They suggest that since metabolism is a conservative evolutionary feature, the examples found on the extremes of the continuum are responding, by way of adaptation, to a wide variety of morphological, behavioral and ecological pressures and not just predatory pressures.

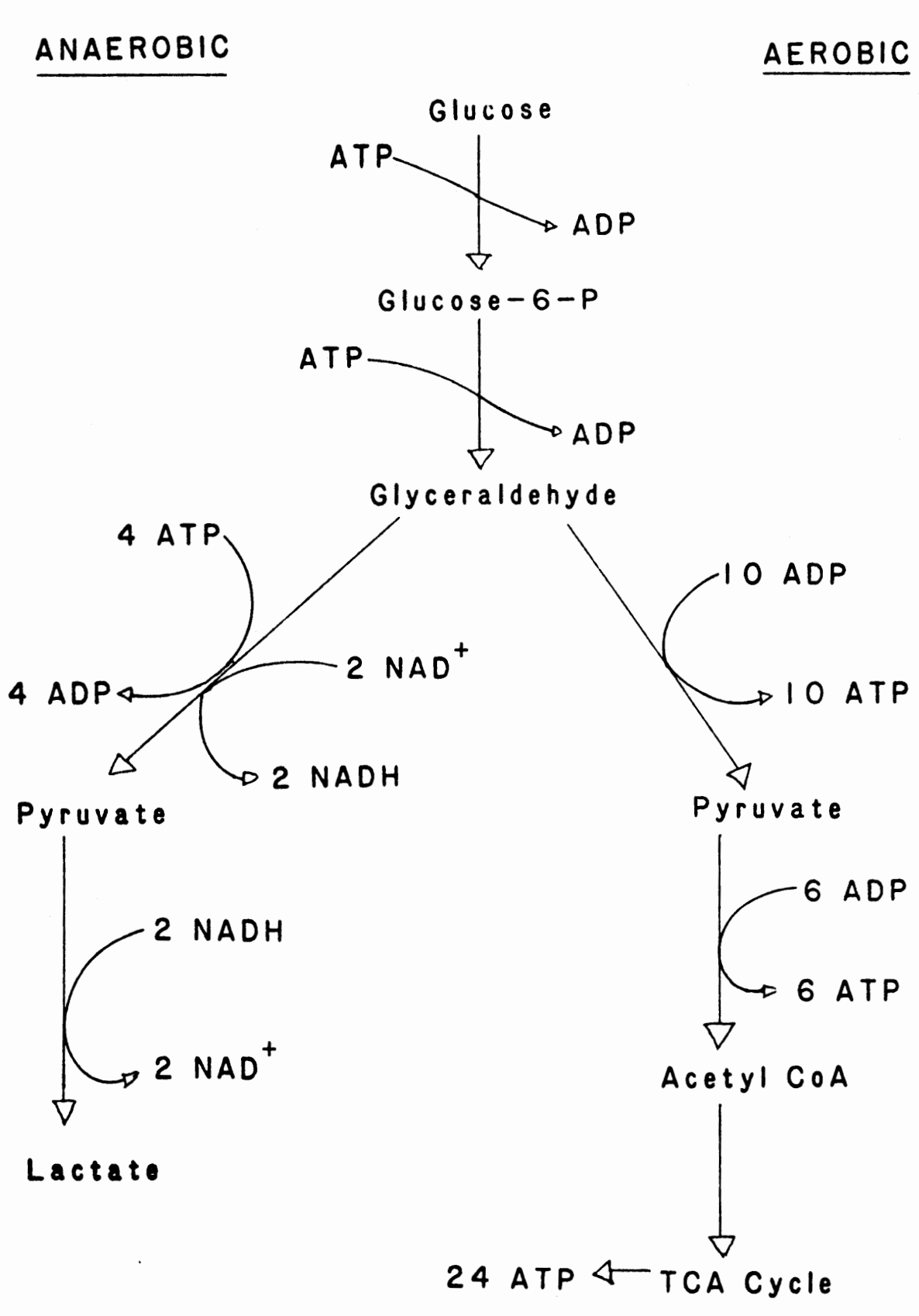
Nevertheless, anuran amphibians provide an excellent model system for the study of aerobic and anaerobic metabolism since individual species can be examined that depend primarily on one mode

of energy generation or the other.

The purpose of this research is to examine some aspects of aerobic and anaerobic metabolism in anurans. Chapter I deals with aerobic metabolism; specifically, the hypoxic limit to $\dot{V}_{O_2}$,max in *Xenopus laevis*. Hypobaric hypoxia was used to examine the physiology of oxygen delivery, especially to determine if diffusion of oxygen from the pulmonary air to the alveolar capillaries is a limitation to $\dot{V}_{O_2}$,max. Also in Chapter I is an examination of the effects of chronic hypoxic acclimation on $\dot{V}_{O_2}$,max and several hematological and morphological parameters. Chapter II is an examination of the fate of C^{14}-labelled lactic acid in three species of anurans. *Rana pipiens* is a primarily anaerobic frog, *Bufo americanus* is a primarily aerobic toad and *Xenopus laevis* is intermediate in metabolic strategies, with a high $\dot{V}_{O_2}$,max and high lactate concentrations following exercise.

Figure 1.

Summary of the aerobic and anaerobic metabolic pathways in the catabolism of glucose, stressing energetic requirements and yields.


ANAEROBIC
AEROBIC
Glucose
ATP
ADP
Glucose-6-P
ATP
ADP
Glyceraldehyde
4 ATP
2 NAD+
4 ADP
2 NADH
Pyruvate
2 NADH
2 NAD+
Lactate
10 ADP
10 ATP
Pyruvate
6 ADP
6 ATP
Acetyl CoA
24 ATP
TCA Cycle

CHAPTER I

INTRODUCTION

Oxygen availability can be potentially reduced in aquatic environments, during periods of submergence in hypoxic waters, when burrowed or at high altitudes. Some physiological effects of hypoxia on amphibians have been studied, including effects on heart rate and degree of dilation in cutaneous and skeletal muscle capillaries (Armentrout and Rose, 1971), respiratory patterns (Boutilier and Towes, 1977), and metabolic responses such as lactate levels, liver glycogen levels, and blood sugar (Armentrout and Rose, 1971; Jones and Mustafa, 1973). These studies were concerned with the effect of hypoxia on resting oxygen consumption and did not address its effect on maximal rates of oxygen consumption. They were also concerned only with the effects of acute hypoxia (or anoxia), i.e. exposures were less than one day.

Maximal oxygen consumption ($\dot{V}_{O_2}$,max) should be more affected by hypoxia than resting oxygen consumption since availability of oxygen may become rate limiting for maximum aerobic metabolism at a higher critical oxygen tension. Withers (1980) has demonstrated this higher critical oxygen tension for $\dot{V}_{O_2}$,max than $\dot{V}_{O_2}$,rest in lungless salamanders. The critical oxygen tension will be equal to ambient oxygen tension if maximal oxygen consumption is normally limited by the rate of transport of oxygen from pulmonary air to blood, i.e.

$\dot{V}_{O_2}$,max would decrease with a decline in oxygen tension. This first diffusive step of oxygen delivery is dependent on the diffusion gradient (i.e. oxygen tension of pulmonary air to blood) and the diffusive capacity. Hillman and Withers (1979) have concluded that respiratory surface area, "does not impose a maximum limit on gas exchange in anuran amphibians".

Hematological parameters such as hematocrit and hemoglobin concentration are known to increase during chronic hypoxic exposure. Ventricle and lung size could increase in chronic hypoxia in order to increase oxygen delivery. Increases in these parameters should also serve to increase $\dot{V}_{O_2}$,max.

This investigation is the first study to evaluate the effects of hypoxia on V_{O_2},max in anuran amphibians and attempts to induce physiological adaptations to chronic hypoxia in *Xenopus laevis* by exposing them to decreased oxygen tensions for a period of two weeks. After this period of acclimation, V_{O_2},max and several hematological and morphological parameters were measured.

MATERIALS AND METHODS

Chronic Hypoxia

A group of eight frogs was acclimated for two weeks at a barometric pressure of 500 torr (ambient P_{O_2} = 100 torr). The $\dot{V}_{O_2}$,max was measured (see below) for this group after acclimation and compared to a control group. The acclimated group was then further exposed for two weeks to barometric pressures of 100 torr (ambient P_{O_2} = 20 torr).

Acclimation was accomplished by placing two frogs in each of four

Nalgene seven liter desiccators containing one liter of water. The desiccators were evacuated to the desired pressure (500 or 100 torr). Controls were placed in screened plastic containers with two animals each in one liter of water. All animals were kept in the dark and the air flushed and low pressure restored daily for two weeks.

It can be calculated that the frogs used approximately 60% of the available O_2 in 24 hours at the acclimation pressure of 100 torr, given a standard $\dot{V}_{O_2}$ of 0.1 ml $O_2 \cdot g^{-1} hr^{-1}$ (Hillman and Withers, 1979). The level of hypoxic stress was, therefore, actually less than 100 torr.

Maximal Oxygen Consumption

The $\dot{V}_{O_2}$,max was determined by the method previously described by Seymour (1973) and Hillman (1976). The procedure consists of placing the animal in a closed metabolic chamber (volume = 450 ml) and evacuating the chamber to the desired experimental P_{O_2}. The chamber was then manually rotated to flip the animal on its dorsum, thereby constantly eliciting the righting reflex. A 15 to 20 ml air sample was withdrawn into a 50 ml syringe after an activity bout of 5 minutes; CO_2 and water vapor removed with Ascarite and Drierite, respectively and oxygen content determined with a Beckman OM-14 polarographic oxygen analyser. Temperature of acclimation and activity was 19°C.

Hematology and Morphology

Hematological and morphological values were measured for the 100 torr acclimated and control *Xenopus laevis*. The animals were pithed, ventricle and lungs removed, blotted dry and weighed to the

nearest 0.1 mg. A blood sample was taken from the ventricle in a heparinized capillary tube which was centrifuged at 3400 RPM for 5 minutes. Hematocrit is the percent packed red cells. Hemoglobin content was determined by the cyanomethemoglobin method and red blood cell count was measured on a Coulter counter model Z_{BI}. Mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were determined as:

$$MCV = \frac{\text{Hematocrit (\%)} \times 10}{\text{RBC count (in millions)}}$$

$$MCH = \frac{\text{Hemoglobin (g}\cdot 100\ \text{ml}^{-1}) \times 10}{\text{RBC count (in millions)}}$$

$$MCHC = \frac{\text{Hemoglobin (g}\cdot 100\ \text{ml}^{-1}) \times 100}{\text{Hematocrit (\%)}}$$

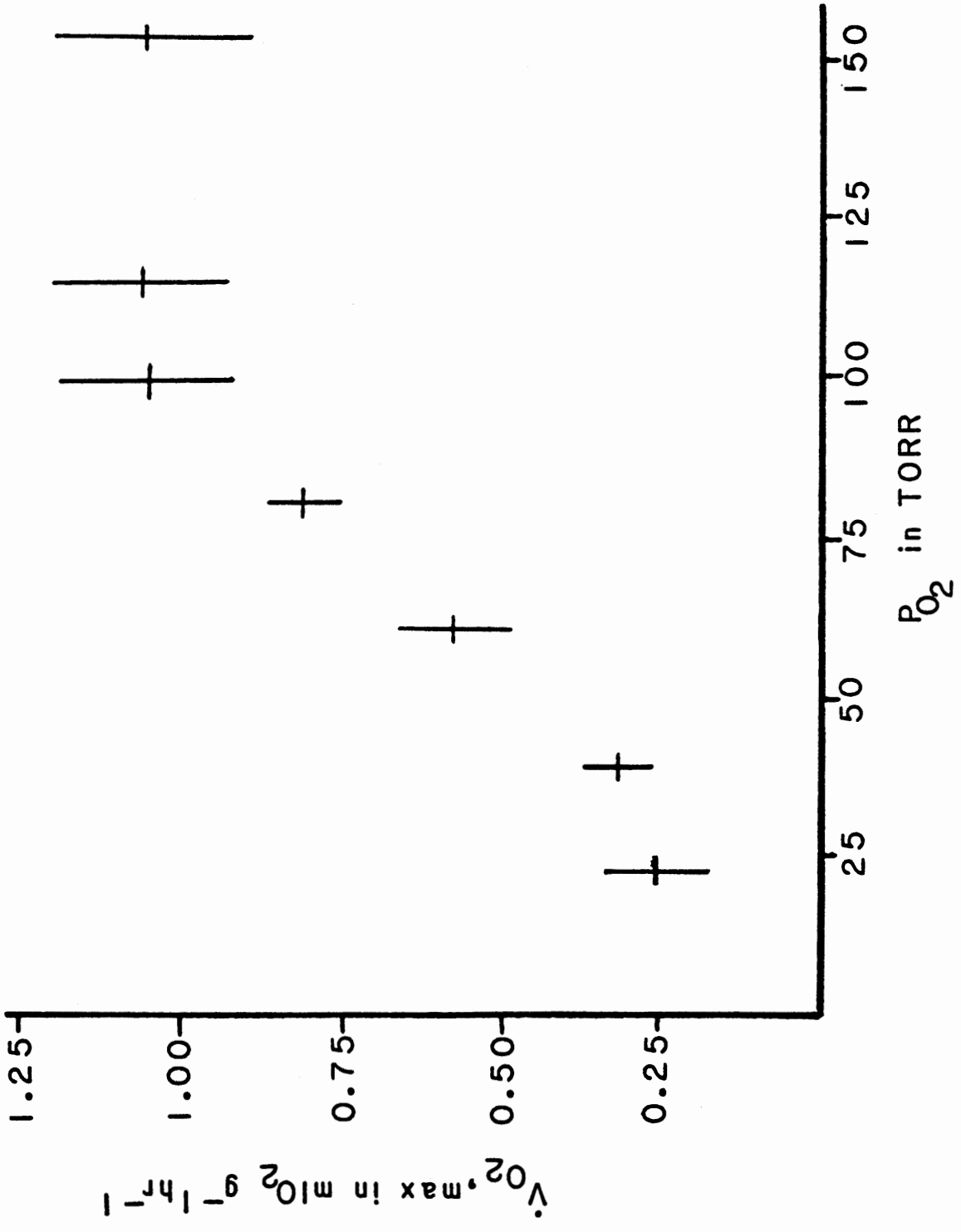
RESULTS

Maximal Oxygen Consumption ($\dot{V}_{O_2}$,max)

The effect of hypoxia on $\dot{V}_{O_2}$,max in control and chronically acclimated *Xenopus laevis* is shown in Figure 2. The $\dot{V}_{O_2}$,max was 1.10 ± se 0.07 ml $O_2 \cdot g^{-1} hr^{-1}$ (n=15) at normal ambient P_{O_2} of 159 torr and was independent of ambient P_{O_2} above approximately 100 torr. The $\dot{V}_{O_2}$,max was 1.05 ± 0.045 ml $O_2 \cdot g^{-1} hr^{-1}$ (n=36) for all frogs at P_{O_2} above the critical P_{O_2} of 100 torr. The $\dot{V}_{O_2}$,max decreased significantly at P_{O_2} less than 100 torr and was highly correlated with ambient P_{O_2}; $\dot{V}_{O_2}$,max = 0.084 (± se 0.050) + 0.0023 (± se 0.00017)

Figure 2.

Maximal oxygen consumption rates ($\dot{V}_{O_2}$,max) in ml $O_2 \cdot g^{-1} hr^{-1}$ versus P_{O_2} in torr for chronic hypoxia acclimated and control *Xenopus laevis*. Points are means, horizontal and vertical lines are two standard errors.


1.25
1.00
0.75
0.50
0.25
$\dot{V}_{O_2,max}$ in $ml O_2\ g^{-1} hr^{-1}$
25
50
75
100
125
150
P_{O_2} in TORR

x P_{O_2} (r^2 = 0.79; n=51). Two animals died in the 100 torr acclimation period.

Hematology and Morphology

The effect of chronic hypoxia on hematological parameters is shown in Table I. There was a significant increase in RBC count, hematocrit, hemoglobin and MCH but no significant change in MCV or MCHC. There was no significant difference in ventricle mass or lung mass for the two groups.

DISCUSSION

The $\dot{V}_{O_2}$,max measured for *Xenopus laevis* at ambient P_{O_2} of 100-159 torr (1.05 ± se 0.045 ml $O_2 \cdot g^{-1} hr^{-1}$) is in general agreement with that of Hillman and Withers (1979) and is approximately ten times resting $\dot{V}_{O_2}$ for *Xenopus laevis* (Hillman and Withers, 1979). The $\dot{V}_{O_2}$,max declined at P_{O_2} less than 100 torr. Therefore, the critical oxygen tension (P_c) for $\dot{V}_{O_2}$,max in *Xenopus laevis* is about 100 torr. A similar P_{O_2} dependence has been determined for plethodontid salamanders by Withers (1980) in which the critical P_{O_2} was 110 torr, for *Bufo cognatus* and *Rana pipiens* in which the critical P_{O_2} was about 80 torr (Withers and Hillman, 1982b) and for aquatic salamanders in the critical P_{O_2} was also 80 torr (Ultsch, 1973).

A P_{O_2} of 100 torr corresponds to about 3,000 meters in altitude; this exceeds the altitudinal distribution of amphibians (Hock, 1964). It therefore seems unlikely that $\dot{V}_{O_2}$,max is limited by altitudinally induced hypoxia. These results along with those of

TABLE I

Hematological parameters for control and 100 torr chronic hypoxia acclimated *Xenopus laevis*. Values are mean ± standard error. Asterisk indicates significant difference ($P<0.025$).

TABLE I

HEMATOLOGICAL PARAMETERS FOR CONTROL AND 100 TORR CHRONIC HYPOXIA ACCLIMATED *XENOPUS LAEVIS*. VALUES ARE MEANS ± STANDARD ERROR. ASTERISK INDICATES SIGNIFICANT DIFFERENCE ($P<0.025$).

	Control (n=6)	100 torr acclimated (n=5)
RBC (per mm^3)	154,700 ± 7600	174,800 ± 4300 *
Hct (%)	46.1 ± 1.5	56.4 ± 2.4 *
Hb ($g \cdot 100\ ml^{-1}$)	10.74 ± 0.64	13.88 ± 0.40 *
MCV (μm^3)	3040 ± 97	3440 ± 105
MCH (pg)	694 ± 29	794 ± 17 *
MCHC (%)	23.3 ± 0.66	24.6 ± 0.68

Withers (1980) and Hillman and Withers (1982), indicate that $\dot{V}_{O_2}$,max is not limited by diffusional exchange across the respiratory surface area at P_{O_2} normally encountered. Diffusional exchange is described by Fick's law of diffusion:

$$\dot{V}_{O_2} = D \cdot A \cdot \frac{(P_{A_{O_2}} - P_{a_{O_2}})}{d}$$

where D is the diffusion constant for O_2, A is the respiratory surface area, $P_{A_{O_2}}$ is alveolar P_{O_2}, $P_{a_{O_2}}$ is average pulmonary capillary P_{O_2} and d is the diffusion distance. If $\dot{V}_{O_2}$,max is limited by diffusional exchange across the respiratory surface area, then any decline in ambient P_{O_2} would decrease the oxygen gradient and $\dot{V}_{O_2}$,max would decline. There is, however, no decrease in $\dot{V}_{O_2}$,max with decreased P_{O_2} until the critical P_{O_2} of 100 torr.

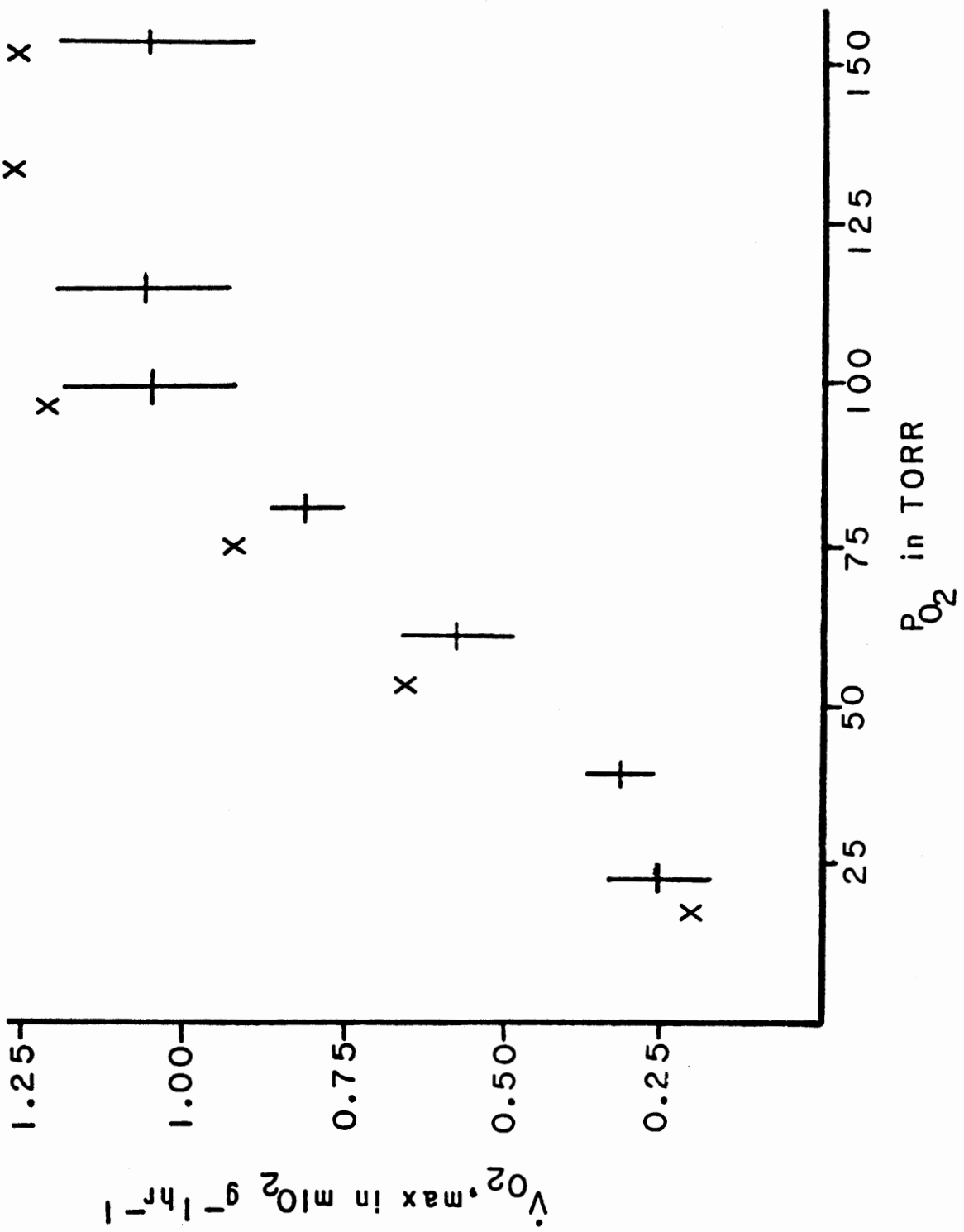
The $\dot{V}_{O_2}$,max declines linearly below the critical P_{O_2} of 100 torr, indicating an O_2 transport limitation at very low P_{O_2}. The intercept of this regression line is -0.084 ± se -0.050 which is not different from zero (intercept t-test = -1.68, degrees of freedom = 49). These data corroborate the findings of Withers and Hillman (1982a) which indicate that there is a cardiovascular and not a respiratory limit to $\dot{V}_{O_2}$,max even at very low P_{O_2} (see Figure 3).

Hematology and Morphology

Packard and Stiverson (1976) observed no change in hemoglobin concentration in anurans along an altitudinal gradient from 1500 to 3000 meters. The results presented here indicate a slight increase

Figure 3.

The $\dot{V}_{O_2}$,max (ml $O_2 \cdot g^{-1} hr^{-1}$) versus ambient P_{O_2}. Graph shows values obtained in this paper (horizontal and vertical bars are two standard errors) and the predicted values (x) of Withers and Hillman (1982).


1.25
1.00
0.75
0.50
0.25
$\dot{V}_{O_2,max}$ in $mlO_2\ g^{-1}\ hr^{-1}$
25
50
75
100
125
150
P_{O_2} in TORR

in hemoglobin concentration in animals acclimated to about P_{O_2} = 20 torr, this increase mainly due to polycythemia. There was also a slight increase in MCH, however, which might indicate that the new population of cells may have had a higher concentration of hemoglobin. The significant aspect of this moderate increase in hemoglobin concentration is that it was correlated with an increase in $\dot{V}_{O_2}$,max at the very lowest P_{O_2} (P_{O_2} = 38 torr). This is consistent with the model of Withers and Hillman (1982a) which predicts that $\dot{V}_{O_2}$,max will increase with an increase in blood hemoglobin. Individual variability at higher P_{O_2} makes it impossible to detect small increases in $\dot{V}_{O_2}$,max predicted by the model.

There was no significant change in lung and ventricle mass, indicating that there was no hypertrophy of these organs as a response to hypoxia.

CHAPTER TWO

INTRODUCTION

Lactic acid accumulation has generally been considered to be an indication of the level of anaerobic metabolism (Bennett and Licht, 1974; Hutchison and Miller, 1979a; Hutchison and Miller 1979b; Preslar and Hutchison, 1978; Hillman and Withers, 1979; Carey, 1979b; Gleeson, 1980; Gatz and Piiper, 1979; Putnam, 1979b) and has been measured during activity in virtually every class of vertebrates (Wardle, 1978; Gleeson, 1980; Hutchison and Miller, 1979a; Taigen et al., 1982; Bartholomew et al., 1976; Dean and Goodnight, 1964; Gatten, 1975; Hermansen and Vaage, 1977; Issekutz et al., 1976).

A major effect of the accumulation of lactic acid is the concomitant decrease in tissue and plasma pH. This disruption of the acid-base balance of the tissue can eventually incapacitate the muscle and has been implicated as the cause of fatigue (Hermansen and Osnes, 1972; Mainwood and Worsley-Brown, 1975). Putnam (1979b), however, has shown that behavioral recovery in *Xenopus laevis* and *Rana pipiens* can be accomplished in both the intact organism and isolated gastrocnemius muscle without lactate disappearance or recovery from pH imbalance. Decreased pH can, however, have a marked effect on many systems including oxygen delivery due to the Bohr effect, enzyme function and blood bicarbonate buffer system.

The fate of lactic acid is unclear, despite the plethora of data

available concerning its production, levels during activity and adverse effects. Original studies on the fate of lactate on isolated frog muscle indicated that about 80% of the lactate was reconverted to glycogen and 20% oxidized aerobically (Hill, 1922). Later results using intact rats fed ^{11}C - labelled lactate revealed that from 10-20% of the lactate was oxidized and 21-32% of the lactate was converted to liver glycogen (Conant et al., 1941; Vennesland et al., 1942), depending on which carbon atoms were labelled.

The Cori cycle is the most widely accepted model of lactate metabolism. According to the Cori cycle, lactate diffuses from the muscle to the blood and is transported to the liver where it is reconverted to glucose and stored as glycogen (Guyton, 1981). This resynthesis is energetically expensive, requiring 6 ATP to convert 2 lactate molecules to one glucose molecule, whereas the original anaerobic yield of glucose catabolism to lactate was only 2 ATP (Lehninger, 1975). An advantage of glucose resynthesis in mammals is that it restores acid-base balance very rapidly. In lower vertebrates, however, very high levels of lactate are accumulated and remain for a considerable time, up to 36 hours (Withers and Hillman, 1981). Consequently acid-base imbalance may also be prolonged for up to 36 hours.

Recent studies concerning the fate of lactic acid after activity, essentially all on mammals (Drury and Wick, 1956; Brooks et al., 1973; Issekutz et al., 1976; Hermansen and Vaage, 1977; McLane and Holloszy, 1979; Brooks and Gaesser, 1980) have led to the conclusion that the Cori cycle cannot account for either lactate disappearance or glycogen

synthesis and suggest that lactate is either mainly converted to glycogen in the muscle itself (Hermansen and Vaage, 1977; McLane and Holloszy, 1979) or mainly oxidized to CO_2 (Brooks and Gaesser, 1980; Drury and Wick, 1956). Clearly the controversy over the metabolic fate of lactate in mammals is not settled, and much more research is needed.

Anaerobic metabolism is of more routine significance in the lower vertebrates, yet there is very little data regarding how these animals metabolize lactate. What little has been published is mainly on isolated frog muscle (Bendall and Taylor, 1970) or fish (Hochachka, 1961). Cushman et al. (1976), in one of the few in vivo studies, found that tiger salamanders do not excrete lactate into the aquatic environment but gave no indication as to its fate.

The purpose of this investigation is to determine, using ^{14}C - labelled lactic acid, the fate of endogenous lactic acid accumulated during activity in anurans. The main concerns were; are the labelled carbon atoms incorporated into specific tissues; how much of the lactate is oxidized aerobically; and how much, if any, of the lactate is excreted directly to ameliorate the acid-base disturbance. Three species of anurans were studied, each having a different mode of energy production. *Rana pipiens* is a primarily anaerobic frog; *Bufo americanus* is a primarily aerobic toad; and *Xenopus laevis* is intermediate in metabolic strategies, with a high $\dot{V}_{O_2}$,max during and high lactate concentrations following exercise. Whole body lactate concentrations were measured for *Xenopus laevis* following recovery periods of various durations after exhaustive exercise in order to

establish a time course for lactate removal. Oxygen consumption was also measured over the entire recovery period.

MATERIALS AND METHODS

Lactate Removal Rate and Oxygen Consumption During Recovery

Xenopus laevis were manually stimulated to be active by the method of Seymour (1973) and Hillman (1976). The animals were placed in a closed metabolic chamber (volume = 450 ml) and the chamber manually rotated to keep the animal constantly righting itself, after it had been flipped on its back. The activity bout was 10 minutes. An air sample was withdrawn into a 50 ml syringe following activity. The sample was passed through Drierite and Ascarite to absorb water vapor and carbon dioxide respectively, and oxygen content measured with a Beckman OM-14 polarographic oxygen analyser.

The animals were immediately placed in 50 ml syringes and held in the dark for the recovery period. The air in the syringes was analysed for oxygen content after various time intervals in the manner described above, and the air in the syringes replenished. Values for oxygen consumption ($\dot{V}_{O_2}$) are expressed as ml O_2 $g^{-1}hr^{-1}$.

Animals were sacrificed at various time intervals and whole body lactate concentrations measured. The method for determining whole body lactate was similar to that of Putnam (1978) and Bennett and Licht (1974). Animals were homogenized in 8% perchloric acid (volume = 10 times body mass), centrifuged at 3000 g for 10 minutes and the supernatant filtered. The supernatant was then analysed for lactate with a commercial lactic acid kit (Sigma Lactic Acid Kit, No. 826-UV).

This assay is based on the enzymatic conversion of lactate to pyruvate which yields an increase in absorbance at 340 nm due to the reduction of NAD^+. Optical density at 340 nm was determined with a Perkin-Elmer model 124D spectrophotometer.

Fate of C^{14}-Lactate

The fate of lactic acid after exercise was examined for *Xenopus laevis*, *Rana pipiens* and *Bufo americanus* using exogenous administration of C^{14}-lactate.

The animals were injected, following exercise, in the dorsal lymph sac with 2 µCi of universally labelled C^{14}-lactate ($C^{14}H_3C^{14}HOH-C^{14}OOH$) obtained from New England Nuclear (specific activity, 165.2 mCi/mmol). The animals were then placed in 50 ml of water for 10 minutes. This water was then sampled (50 µl) and analysed for C^{14}-lactate to determine the extent of possible leakage of C^{14}-lactate from the injection site. The animals were then placed in a chamber with 100 ml (for *X. laevis* and *R. pipiens*) or 50 ml (for *B. americanus*) of water and allowed to recover undisturbed. Recovery times were chosen in accordance with the observed values for whole body lactate removal (8 hours for *X. laevis*, see results) and literature values for *R. pipiens* (8 hours; Hutchison and Turney, 1975) and *B. americanus* (3 hours; Hutchison and Miller, 1979a).

Ambient air was pumped through the chamber during the recovery period, and excurrent air was bubbled through two CO_2 traps, each consisting of 50 ml of saturated KOH. Sampling of the KOH at regular time intervals throughout recovery and analysis for β-emission yielded

a quantitative measure of total $C^{14}O_2$ expired by the animal.

At the end of the recovery period, the animals were weighed, then doubly-pithed and a blood sample obtained from the ventricle in a heparinized capillary tube. The blood sample was centrifuged for 5 minutes to obtain a 20 µl plasma sample which was placed directly into 10 ml of Aquasol-2 scintillation cocktail (New England Nuclear). The animal was then dissected and representative tissues and organs weighed to the nearest 0.1 mg. Samples included muscle (gastrocnemius or posterior thigh muscles), ventricle, lung, liver, skin and gastrointestinal tract. The samples were digested with 0.2 ml concentrated perchloric acid and 0.4 ml 80% hydrogen peroxide at 60°C in an agitating water bath for 60 minutes. The resulting digest was cooled and 10 ml Aquasol-2 scintillation cocktail added. Samples were analysed for β-emission using a microprocessor controlled Beckman LS-9000 liquid scintillation counter. The machine was standardized with Beckman C^{14} standards and the counting time was 60 minutes or one 2 S% error. Results were disintegrations per minute (DPM), converted from the counts per minute (CPM). Details of the counting program, counting efficiency and DPM calculation are given in Appendix A.

RESULTS

Oxygen Consumption Rates and Lactate Concentrations During Recovery

The results obtained for the maximal rate of oxygen consumption ($\dot{V}_{O_2}$,max) and the rate of oxygen consumption during recovery ($\dot{V}_{O_2}$,rec) are given in Figure 4. The $\dot{V}_{O_2}$,max was 1.09 (± se 0.14) ml $O_2 \cdot g^{-1} hr^{-1}$ (n=16). The $\dot{V}_{O_2}$ declined rapidly after activity and was at a standard

$\dot{V}_{O_2}$ of 0.1 ml $O_2 \cdot g^{-1} hr^{-1}$ at 120 minutes after activity and remained at this level throughout the six hour recovery period.

The results for whole body lactate concentrations are given in Figure 5. The regression line for lactate removal was [lactate] = -0.26 (± se -0.05) x time + 113.15 (± se 7.49); (r^2 = 0.56; n=23). Maximal lactate levels were 128.36 (± se 13.50; n=7) immediately after activity (t_{rec} = 0 and 15 min). Resting lactate levels of 10 mg · 100 ml^{-1} (Putnam, 1979b) were not attained by the end of the six hour recovery period, but were extrapolated from the regression line to be attained at t_{rec} = 8 hours.

Fate of Lactic Acid

Results for the fate of lactic acid were calculated as the percentage of the injected ^{14}C-labelled lactic acid DPM minus the DPM counted in the 10 minute post-activity recovery water (to account for leakage from the injection site). Results are given as percent DPM (%DPM) and percent DPM per gram (%DPM·g^{-1}) and are summarized in Table II.

The percentage of label recovered as CO_2 is presented in Figure 6 for *R. pipiens* and in Figure 7 for *B. americanus*. *R. pipiens* oxidized approximately 4% of the injected label and *B. americanus* oxidized approximately 2% of the injected label.

The liver, lung, ventricle and blood contained significantly more lactate, both in absolute amounts (%DPM) and mass specific amounts (%DPM·g^{-1}), in *B. americanus* than in *R. pipiens*, whereas the muscle of *R. pipiens* contains more C^{14} activity than the muscle of

B. americanus, in both %DPM and %DPM·g^{-1}.

DISCUSSION

Oxygen Consumption Rates and Lactate Disappearance During Recovery

The $\dot{V}_{O_2}$ of *Xenopus laevis* dropped rapidly during recovery and reached standard levels in all animals within two hours of the end of activity. The rate of lactate disappearance does not coincide with the $\dot{V}_{O_2}$. Resting levels of lactate are not achieved after six hours of recovery. By extrapolation of the regression line, time of recovery to resting rates of lactate is eight hours.

Hutchison and Miller (1979b) report return to resting lactate levels in *Xenopus laevis* at nine hours. Differences in the stimulation technique (electrical vs. manual stimulation) may, in part, account for the difference. Hutchison and Miller (1979b) also obtained higher whole body lactate concentrations of 222 mg·100 ml^{-1}. This is also possibly due to electrical stimulation (Putnam, 1979b; Hillman et al., 1979). Putnam (1979b) obtained higher whole body lactate concentrations (213 mg·100 ml^{-1}) using manual stimulation. The technique used was forced swimming, a regimen which possibly involves more muscle groups and possibly led to fatigue more rapidly.

Fate of Lactic Acid

The liver, lung, ventricle and blood contained significantly more lactate per gram in *Bufo americanus* than *Rana pipiens*, while muscle contained more lactate per gram in *R. pipiens* than *B. americanus*. *Rana pipiens* depends primarily on anaerobic metabolism

TABLE II

The fate of lactic acid in some anuran amphibians. Values are means ± standard error for *Rana pipiens* (n=4) and *Bufo americanus* (n=4). Values for *Xenopus laevis* for 4 hour (n=2) and 8 hour (n=2) recovery are means.

TABLE II

	%DPM				%DPM·g^{-1}			
	R. pipiens	*B. americanus*	*X. laevis* (4 hr)	*X. laevis* (8 hr)	*R. pipiens*	*B. americanus*	*X. laevis* (4 hr)	*X. laevis* (8 hr)
Ventricle	0.07 ± 0.01	0.23 ± 0.06	0.13	0.11	2.06 ± 0.32	3.59 ± 0.25	13.0	6.95
Lung	0.14 ± 0.04	0.34 ± 0.03	0.21	0.15	1.64 ± 0.41	2.39 ± 0.18	12.3	4.59
Liver	0.76 ± 0.12	1.54 ± 0.28	2.00	1.07	2.46 ± 0.35	4.21 ± 0.29	8.71	7.32
G.I. Tract	0.62 ± 0.16	1.21 ± 0.24	1.97	1.48	1.44 ± 0.29	2.07 ± 0.36	8.31	5.32
Skin	4.66 ± 0.62	5.18 ± 0.80	5.52	5.65	2.31 ± 0.43	2.65 ± 0.17	12.9	10.4
Muscle	37.6 ± 2.51	17.9 ± 3.63	25.9	31.6	5.56 ± 0.42	3.42 ± 0.29	9.83	11.8
Blood	3.22 ± 1.00	6.53 ± 0.65	6.3	2.94	1.89 ± 0.32	3.67 ± 0.30	16.5	8.06
Oxidized	4.07 ± 0.60	1.91 ± 0.09	0.31	0.30				
H_2O	6.54 ± 0.63	6.69 ± 3.70	16.7	11.4				

Figure 4.

The rate of oxygen consumption (V_{O_2}) in ml $O_2 \cdot g^{-1} hr^{-1}$ during recovery from exhaustive exercise versus time of recovery.

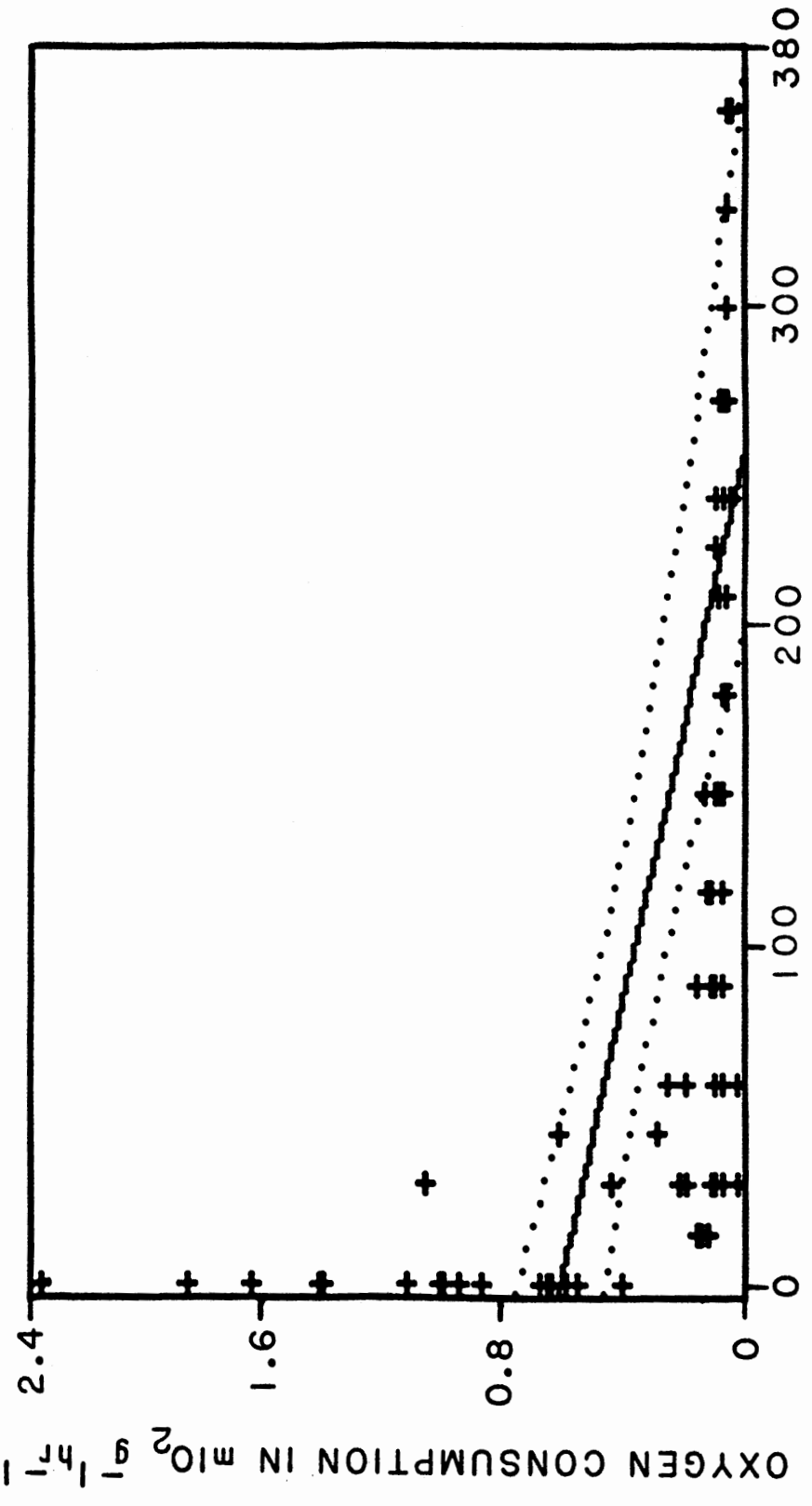

OXYGEN CONSUMPTION IN $ml O_2\ g^{-1}\ hr^{-1}$
2.4
1.6
0.8
0
0
100
200
300
380
TIME OF RECOVERY IN MINUTES

Figure 5.

Whole body lactate concentrations ($mg \cdot 100\ ml^{-1}$) during recovery from exhaustive exercise versus time of recovery (in minutes).

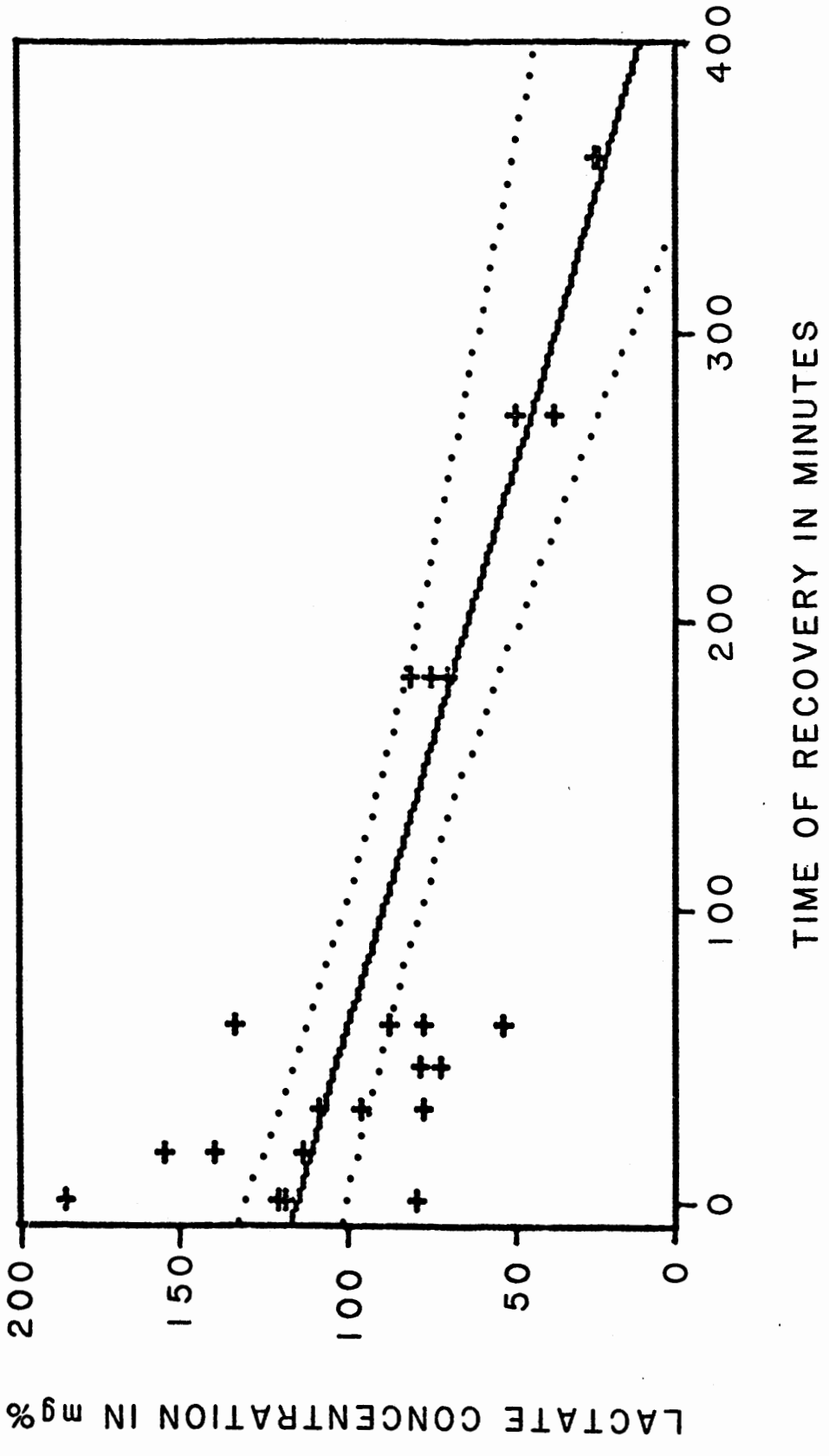

LACTATE CONCENTRATION IN mg%
200
150
100
50
0
0
100
200
300
400
TIME OF RECOVERY IN MINUTES

Figure 6.

The percentage of C^{14} label injected (%DPM) recovered as expired C^{14} O_2 versus time of recovery (hours) for *Rana pipiens*.

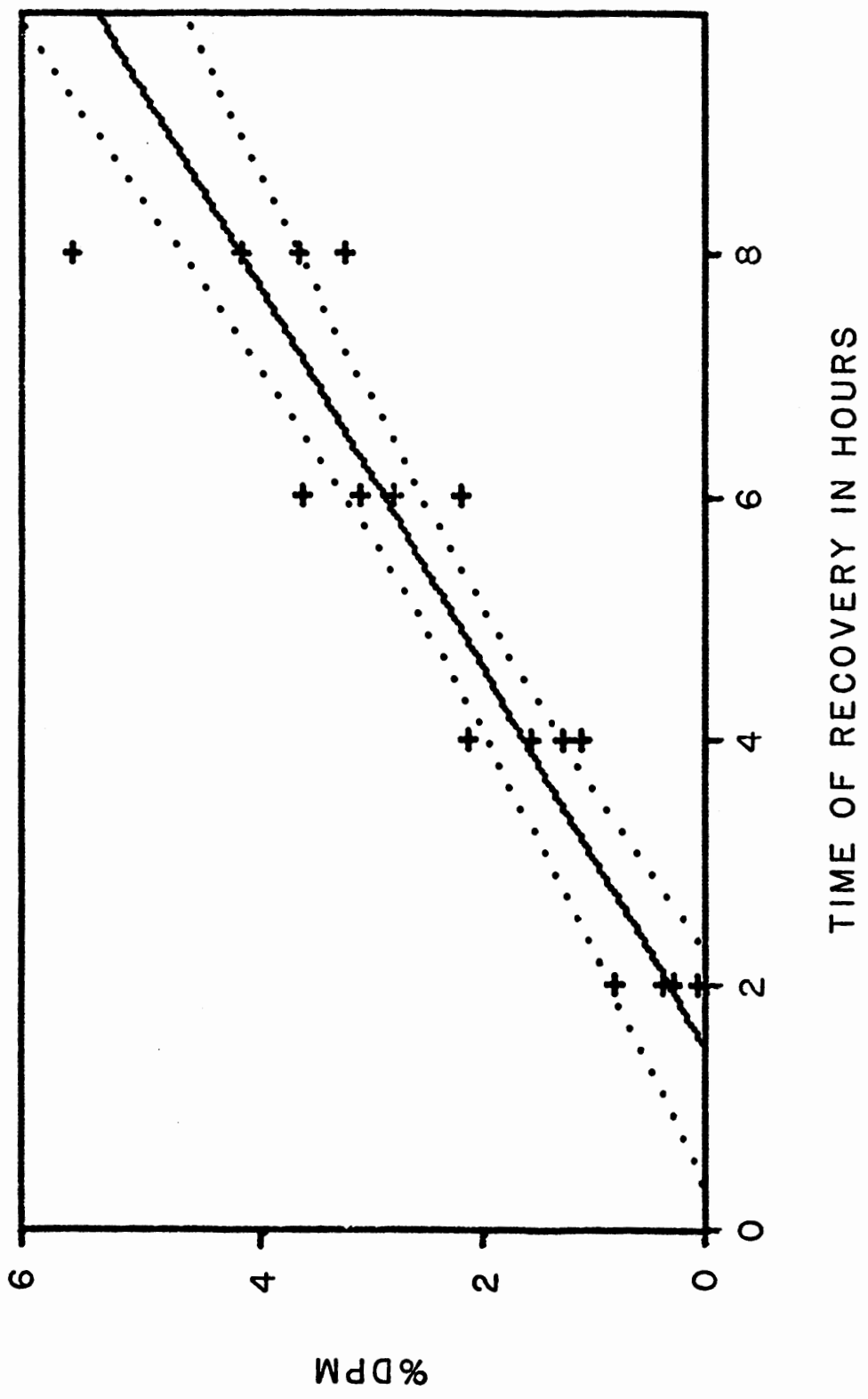
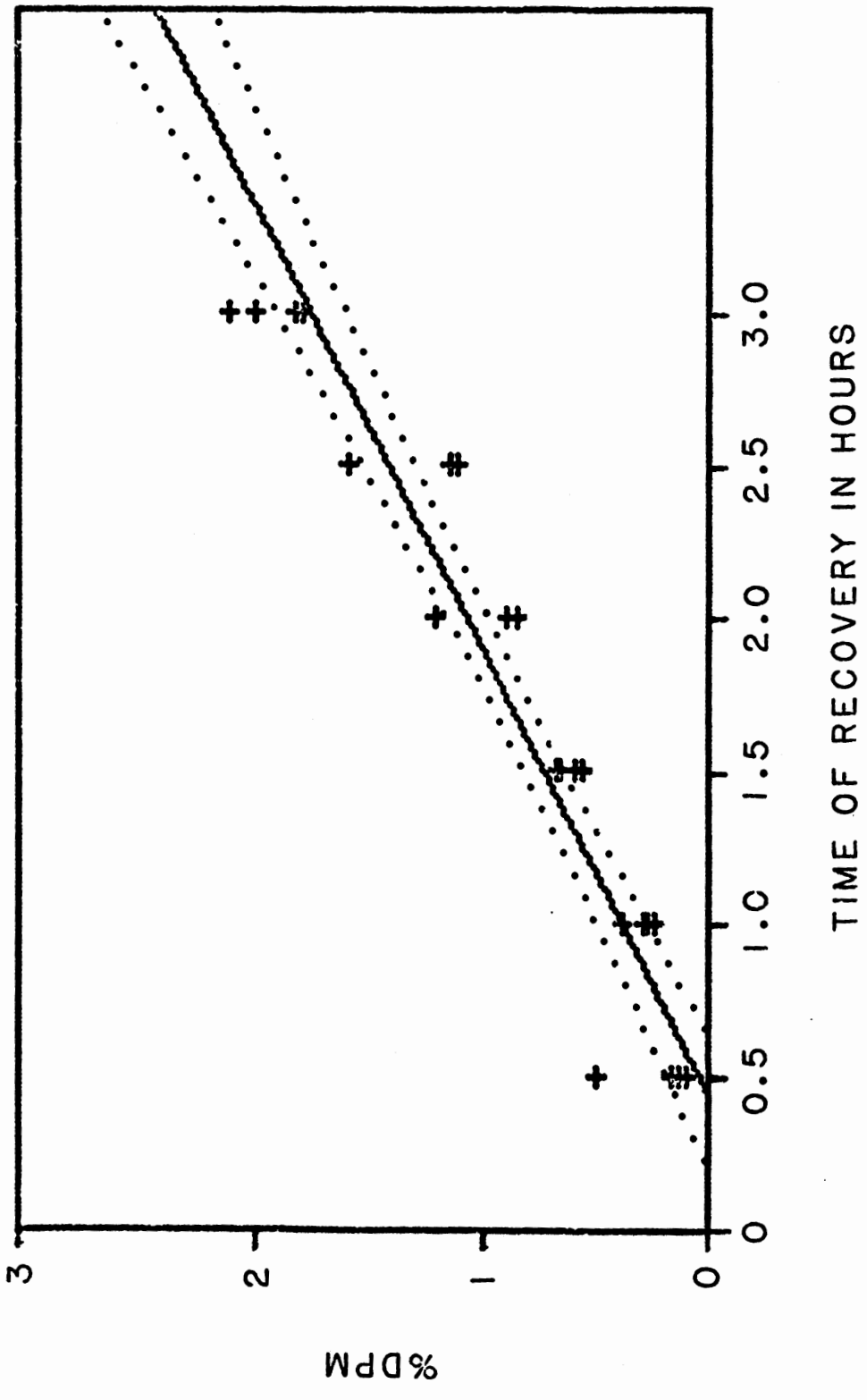

%DPM
0
2
4
6
0
2
4
6
8
TIME OF RECOVERY IN HOURS

Figure 7.

The percentage of C^{14} label injected (%DPM) recovered as expired C^{14} O_2 versus time of recovery (hours) for *Bufo americanus*.


%DPM
3
2
1
0
0
0.5
1.0
1.5
2.0
2.5
3.0
TIME OF RECOVERY IN HOURS

during activity. It is necessary, therefore, that a readily available supply of glucose is available in the muscle tissue for burst activity. Although the experiments presented here do not include biochemical assays to determine the nature of the molecules that the labelled carbon is incorporated into, it is likely that a significant portion is in glycogen. Previous studies of mammalian systems have indicated that a large percentage of endogenous lactate is converted to glycogen (Hermansen and Vaage, 1977; McLane and Holloszy, 1979). Bendall and Taylor (1970), working with isolated frog sartorius muscle, concluded that almost 80% of the endogenous lactate was converted to glycogen, either within the muscle or by another tissue such as the liver in the Cori cycle. The animal, therefore, has adequate muscle glucose for subsequent burst anaerobic metabolism. Storing the carbons from lactic acid in other compounds such as fatty acids or proteins would be of little value for a principally anaerobic species such as *R. pipiens*, because fat and protein are not suitable substrates for anaerobiosis. When large fat bodies were found during dissection, they were dissolved in 10 ml of scintillation cocktail and analysed for C^{14}. Very little of the C^{14} (approximately 0.8 $\%DPM \cdot g^{-1}$) was incorporated into fat.

Less C^{14} activity was found in the muscle of *B. americanus* and more activity was found in the lung, liver, ventricle and blood. This species has a primarily aerobic metabolic strategy, therefore the demand for readily available muscle glucose reserves is less. Apparently more of the lactate is metabolized by tissues other than the muscle in *B. americanus*.

Aerobic oxidation accounted for approximately 4% of the lactate in *R. pipiens* and about 2% in *B. americanus*. These are much lower than reported values for the fate of lactate, which range from 10% expired in rats (Vennesland et al., 1942) to 80-90% expired in rabbits (Drury and Wick, 1956). Bendall and Taylor (1970) found that isolated frog sartorius muscles oxidized 20% or less of the lactate that accumulated during activity. Oxidation of lactate directly would, a priori, appear to be a likely fate of lactate. The lactate removal rate is slow, and the $\dot{V}_{O_2}$ is low in anurans compared to mammals, after anaerobic activity. Resynthesis of one molecule of glucose from two molecules of lactate is energetically expensive, requiring 6 ATP, therefore direct oxidation appears energetically favorable.

Lactate does not appear to be oxidized by these anurans, however, and it appears that lactate is not preferentially metabolized. The animals were injected with 0.012 μmoles of labelled lactate. In a 20 g frog which accumulated 128 mg·110 ml^{-1} of lactate, the ratio of unlabelled to labelled lactate is approximately 2.4×10^4 : 1. Over the recovery period, the frog consumed about 16 ml of oxygen (0.2 ml $O_2 \cdot g^{-1} hr^{-1}$ for 8 hours). Assuming an RQ of 1, 16 ml or 710 μmoles of CO_2 are produced. The mean values for μmoles of $C^{14}O_2$ produced for *R. pipiens* was 4.4×10^{-4}. The ratio of unlabelled to labelled CO_2 was, therefore, 1.6×10^6 : 1. This value is 67 times greater than the unlabelled to labelled lactate ratio. It, therefore, seems unlikely that the exogenous, labelled lactate is simply equilibrating with the general body carbon pool. Lactate appears to be preferentially removed by some pathway other than aerobic metabolism;

the endogenous lactic acid is presumably removed in the same manner.

Sample sizes for the two groups of *X. laevis* are, of course, too small to be of significance; nevertheless, some trends are suggested which are of interest. After 4 hours of recovery, over one-half of the recovered label is found in the ventricle, lung, liver and blood. All of these tissues decline in %DPM·g^{-1} after 8 hours recovery, and there is a slight increase in the muscle %DPM·g^{-1}. In *X. laevis* the amount of C^{14} recovered as CO_2 is negligible (less than 0.5 %DPM); this is consistent with other *X. laevis* analysed in preliminary experiments not reported here. It is, therefore, apparent that *X. laevis* oxidizes almost no lactate during recovery.

There has been very little data concerning the fate of lactate, especially in amphibians. It has generally been assumed on energetic grounds, that the majority of the lactate accumulated after activity was oxidized (Preslar and Hutchison, 1979; Withers and Hillman, 1981). The data presented here indicate that oxidation contributes very little to the removal of lactate.

The carcass remaining after the dissection was dissolved in saturated KOH in order to determine the total recovery of the C^{14} activity. Total recovery was determined as the sum of the tissue samples %DPM, the oxidized $C^{14}O_2$ %DPM, the recovery waters %DPM, the carcass %DPM and an extrapolated estimation of the fluid lost in dissection (based on the difference in initial body mass and the sum of the tissue masses after dissection). Total recovery of the C^{14} activity was 80.1 ± 5.8% for *R. pipiens* and 90.3 ± 13.0% for *B. americanus*. Mahin and Lofberg (1966) report that the digestion

technique used here may be accompanied by a 10% loss of C^{14} activity due to the production of $C^{14}O_2$.

REFERENCES

Armentrout, D. and F.L. Rose (1971) Some physiological responses to anoxia in the Great Plains Toad, *Bufo cognatus*. Comp. Biochem. Physiol. 39A: 447-455.

Bartholomew, G.A. and A.F. Bennett and W. Dawson (1976) Swimming, diving and lactate production in the marine iguana, *Amblyrhynchus cristatus*. Copiea. 709-720.

Bendall, S.R. and A.A. Taylor (1970) The Meyerhof Quotient and the synthesis of glycogen from lactate in frog and rabbit muscle. Biochem. J. 118: 887-893.

Bennett, A.F. (1978) Activity metabolism of the lower vertebrates. Ann. Rev. Physiol. 400: 447-469.

Bennett, A.F. and P. Licht (1973) Relative contributions of anaerobic and aerobic energy production during activity in amphibia. J. Comp. Physiol. 87: 351-360.

Bennett, A.F. and P. Licht (1974) Anaerobic metabolism during activity in amphibians. Comp. Biochem. Physiol. 48A: 319-327.

Boutilier, R.G. and D.P. Towes (1977) The effect of progressive hypoxia on respiration in the toad, *Bufo marinus*. J. Exp. Biol. 68: 99-107.

Brooks, G.A., K.E. Brauner, and R.G. Lassens (1973) Glycogen synthesis and the metabolism of lactic acid after exercise. Am. J. Physiol. 224: 1162-1166.

Brooks, G.A., and G.A. Gaesser (1980) End points of lactic and glucose metabolism after exhausting exercise. J. App. Physiol. 1057-1069.

Carey, C. (1979a) Effect of constant and fluctuating temperatures on resting and active oxygen consumption of toad, *Bufo boreas*. Oecologia. 39: 201-212.

Carey, C. (1979b) Aerobic and anaerobic energy expenditure during rest and activity in montane *Bufo b. boreas* and *Rana pipiens*. Oecologia. 39: 213-228.

Conant, J.B., R.D. Cramer, A.B. Hastings, F.W. Klemperer, A.K. Solomon, and B. Vennesland (1941) Metabolism of lactic acid containing radioactive carboxyl carbon. J. Biol. Chem. 137: 557-566.

Cushman, J.R., G.C. Packard, and T.V. Boardman (1976) Concentrations of lactic acid in neotenic and transformed tiger salamanders (*Ambystoma tigrinum*) before and after activity. J. Comp. Physiol. 112: 273-281.

Dean, J.M. and C.V. Goodnight (1964) A comparative study of carbohydrate metabolism in fish as affected by temperature and exercise. Physiol. Zool. 37: 280-299.

Drury, D.R. and A.N. Wick (1956) Metabolism of lactic acid in the intact rabbit. Am. J. Physiol. 184: 305-308.

Gatten, R.E., Jr. (1975) Effects of activity on blood oxygen saturation, lactate, and pH in the turtles *Pseudomys scripta* and *Terrapene ornata*. Physiol. Zool. 48: 24-35.

Gatz, R.N. and J. Piiper (1979) Anaerobic energy metabolism during severe hypoxia in the lungless salamander, *Desmognathus fuscus* (Plethodontidae). Respir. Physiol. 38: 377-384.

Gleeson, Todd T. (1980) Metabolic recovery from exhaustive activity by a large lizard. J. Appl. Physiol. 48: 689-694.

Guyton, A.C. (1981) Textbook of Medical Physiology. W.B. Saunders Co. Philadelphia.

Hermansen, L. and J.B. Osnes (1972) Blood and muscle pH after maximal exercise in man. J. Appl. Physiol. 32: 304-322.

Hermansen, L. and O. Vaage (1977) Lactate disappearance and glycogen synthesis in human muscle after maximal exercise. Am. J. Physiol. 233(5): E422-E429.

Hill, A.V. (1922) The mechanism of muscle contraction. Physiol. Rev. 2: 310.

Hillman, S.S. (1976) Cardiovascular correlates of maximal oxygen consumption rates in anuran amphibians. J. Comp. Physiol. 109: 199-207.

Hillman, S.S., V.H. Shoemaker, R. Putnam, and P.C. Withers (1979) Reassessment of aerobic metabolism in amphibians during activity. J. Comp. Physiol. 129: 309-313.

Hillman, S.S. and P.C. Withers (1979) An analysis or respiratory surface area as a limit for activity metabolism in anurans. Can. J. Zool. 57: 2100-2105.

Hochachka, P.W. (1961) The effect of physical training on oxygen debt and glycogen reserves in trout. Can. J. Zool. 39: 767-776.

Hochachka, P.W. (1980) Living Without Oxygen. Harvard University Press, Cambridge, MA.

Hock, R.J. (1964) Animals at high altitude: reptiles and amphibians. p. 841-842. In D.B. Dill (Ed). Handbook of Physiology, Section 4, Adaptations to the Environment. Am. Physiol. Soc., Washington D.C.

Hutchison, V.H. and K. Miller (1979a) Anaerobic capacity of amphibians. Comp. Biochem. Physiol. 63A: 213-216.

Hutchison, V.H. and K. Miller (1979b) Aerobic and anaerobic contributions to sustained activity in *Xenopus laevis*. Respir. Physiol. 38: 98-103.

Hutchison, V.H. and L.D. Turney (1975) Glucose and lactate concentrations during activity in the leopard frog *Rana pipiens*. J. Comp. Physiol. 99: 287-295.

Hutchison, V.H., L.D. Turney, and R.K. Gratz (1977) Aerobic and anaerobic metabolism during activity in the salamander *Ambystoma tigrinum*. Physiol. Zool. 50: 189-202.

Issekutz, B., Jr., W.A.S. Shaw, and A.C. Issekutz (1976) Lactate metabolism in resting and exercising dogs. J. Appl. Physiol. 40(3): 312-319.

Jones, D.R. and T. Mustafa (1973) The lactacid debt in frogs after one hours aphoea in air. J. Comp. Physiol. 85: 15-24.

Lehninger, A.L. (1975) Biochemistry. 2nd Ed. Worth Publishers Inc. New York.

Mahin, D.T. and D.T. Lofberg (1966) A simplified method of sample preparation for determination of tritium, carbon-14 or sulfer-35 in blood or tissue by liquid scintillation counting. Anal. Biochem. 16: 500-509.

Mainwood, G.W. and P. Worsley-Brown (1975) The effects of extracellular pH and buffer concentration on the efflux of lactate from frog sartorius muscle. J. Physiol., London. 250: 1-22.

McLane, Jerry A. and John O. Holloszy (1979) Glycogen synthesis from lactate in the three types of skeletal muscle. J. Biol. Chemistry. 254(14): 6548-6553.

Packard, G.C. and R.K. Stiverson (1976) Blood hemoglobin concentration in chorus frogs (*Pseudocris triseriata*): Relationships to body size and altitude. Am. Midl. Nat. 96: 482-487.

Preslar, A.J. III and V.H. Hutchison (1978) Energetics for activity in the salamander *Amphiuma means*. J. Comp. Physiol. 128: 139-146.

Putnam, R.W. (1979a) The basis for differences in lactic acid content after activity in different species of anuran amphibians. Physiol. Zool. 52: 509-519.

Putnam, R.W. (1979b) The role of lactic acid accumulation in muscle fatigue in two species of anurans, *Xenopus laevis* and *Rana pipiens*. J. Exp. Biol. 82: 35-51.

Putnam, R.W. and A.F. Bennett (1981) Thermal dependence of behavioral performance of anuran amphibians. Anim. Behav. 29: 502-509.

Seymour, R.S. (1973) Physiological correlates of forced activity and burrowing in the spadefoot toad, *Scaphiopus hammondi*. Copeia. 193: 103-115.

Taigen, T.L., S.B. Emerson, and F.H. Pough (1982) Ecological correlates of anuran exercise physiology. Oecologia. 52(1): 49-56.

Ultsch, G.R. (1973) A theoretical and experimental investigation of the relationships between metabolic rate, body size, and oxygen exchange capacity. Respir. Physiol. 18: 143-160.

Vennesland, B., A.K. Solomon, J.M. Buchanan, R.D. Cramer, and A.B. Hastings (1942) Metabolism of lactic acid containing radioactive carbon in the α or β position. J. Biol. Chem. 142: 371-377.

Wardle, C.S. (1978) Non-release of lactic acid from anaerobic swimming muscles of plaice *Pleuronectes platessa* L.: a stress reaction. J. Exp. Biol. 77: 101-155.

Withers, P.C. (1980) Oxygen consumption of plethodontid salamanders during rest, activity and recovery. Copiea 1980(4): 781-787.

Withers, P.C. and S.S. Hillman (1981) Oxygen consumption of *Amphiuma means* during forced activity and recovery. Comp. Biochem. Physiol. 69A: 141-144.

Withers, P.C. and S.S. Hillman (1982a) A computer simulation of maximal oxygen transport rate in anuran amphibians. (M.S.).

Withers, P.C. and S.S. Hillman (1982b) The effect of hypoxia on pulmonary function and maximal rates of oxygen consumption in two anuran amphibians. (M.S.).

APPENDIX A

A copy of the counting program is on the following page. Counting efficiencies were calculated internally by the following formula:

$$\% \text{ efficiency} = \frac{\text{CPM (observed) - background}}{\text{DPM (in sample)}} \times 100$$

Counting efficiency was based on calibration with Beckman C^{14} standards and was determined as:

$$\text{counting efficiency} = A + Bx + Cx^2 + Dx^3$$

where x = H number and A, B, C, D = Quench mode coefficients. Disintegrations per minute (DPM) is then calculated by:

$$\text{DPM} = \frac{\text{CPM (sample) - background}}{\text{counting efficiency}}$$

The error factor $\sigma(\%)$ error is the real error calculated and based on actual counting data:

$$\pm 2\,\sigma(\%) = \pm \frac{200}{\sqrt{N}}$$

Where N is the total number of counts obtained at the time of calculation.

```
PROG  1       USER  1

CNT CH H123   1 TIMES
CSS           1 TIMES
SCR =2/3
AQC =YES
RCM =NO
CALC=         4
PST =     60.00 MIN
QCF =         0 HH
CH 1      10.00 2 SIGMA %
             .0 LSR
             .0 BKG
            .00 2 SIGMA B
              0 LL
            397 UL
CH 2      10.00 2 SIGMA %
             .0 LSR
             .0 BKG
            .00 2 SIGMA B
              0 LL
            655 UL
CH 3       1.00 2 SIGMA %
             .0 LSR
             .0 BKG
            .00 2 SIGMA B
            397 LL
            655 UL


SINGLE LABEL DPM-VERS:08/01/76

1. STANDARDS ID: BUCKMAN C14
2. UNK ID: TOAD LACTATE
     UNKNOWN NORMALIZATION FACTOR:        1.00000000
3. DATA CHANNEL: 3
4. QUENCH MODE: H
    BKGRD CONSTANT QUENCH? Y
5. HALF LIFE(DAYS):          0.0000
6. CALCULATE COEFF.? N
7. AVG BKGRD:
    CHAN #3:       13.7900
8. QUENCH COEFFS(A,B,C,D):
          81.889491,-    0.148053,      0.001092,-  0.00000304
9. QUENCH LIMITS(LOW,HIGH):      39.000000, 282.00000000
```