The Journal of Experimental Biology 202, 579–592 (1999)
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JEB1732

LACTATE METABOLISM AND GLUCOSE TURNOVER IN THE SUBTERRANEAN CRUSTACEAN *NIPHARGUS VIREI* DURING POST-HYPOXIC RECOVERY

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Accepted 10 December 1998; published on WWW 3 February 1999

Summary

Glucose and lactate metabolism were studied in a hypoxia-resistant subterranean crustacean, *Niphargus virei*, using an injection of L-[U-^{14}C]lactate and tracer D-[6-^{3}H]glucose either in normoxic conditions or after a 24 h exposure to severe hypoxic. Post-hypoxic animals (H animals) were compared with two treatment groups of normoxic animals. In the first normoxic group (NLL animals), animals were simultaneously injected with labelled and unlabelled lactate to obtain a lactate load similar to that of H animals. In the second normoxic group (N, control animals), animals were only injected with labelled lactate. During a 24 h recovery period, the incorporation of ^{14}C and ^{3}H into glycogen, lactate, glucose, amino acids, lipids and CO_2 was measured.

During recovery, glucose turnover rate was enhanced in H and depressed in NLL compared with N animals. However, when energy expenditure was taken into account, the changes were due only to a reduction of glucose turnover rate by lactate load. It was concluded that gluconeogenesis was not the main source of glyconeogenesis.

Equivalent lactate loading in NLL and H animals resulted in an equivalent enhancement (fivefold) of lactate utilization in both groups when energy expenditure was taken into account. Lactate label incorporation appeared later in glycogen than in glucose, but remained high 24 h after the injection. Since glucose is mainly an extracellular metabolite, this observation may be consistent with the hypothesis of two distinct sites for glycogen restoration in hypogean crustaceans: a gluconeogenic organ (a liver equivalent) and a glyconeogenic organ (a muscle equivalent).

The oxidative pathways of glucose and lactate were depressed in post-hypoxic *N. virei* and to a lesser extent in the NLL group. Since there is no evidence of marked protein utilization, it is postulated that, during recovery, repayment of the O_2 debt relies on an increase in lipid utilization.

During recovery from severe hypoxia or after a lactate load, the subterranean *N. virei* appeared to implement a strategy of lactate removal quite different from that observed in epigean crustaceans, favouring lactate-supported gluco- and glyconeogenesis and rapid glycogen replenishment instead of rapid lactate removal *via* oxidative pathways.

Key words: crustacean, hypogean, epigean, gluconeogenesis, glyconeogenesis, lactate metabolism, glucose turnover, hypoxia, recovery, amphipod, *Niphargus virei*.

Introduction

During the last 20 years, the survival, behaviour, physiology and metabolism of aquatic surface-dwelling (epigean) crustaceans (especially marine decapods) under environmental or functional anaerobiosis have been the subject of a few investigations (for reviews, see Zebe, 1991; Hervant and Mathieu, 1995; Hervant et al., 1996; Malard and Hervant, 1998). It was found that most epigean crustaceans show little tolerance to severe hypoxia or anoxia and use only the basic pathway of glycogen fermentation with lactate as the unique end product.

In contrast, the subterranean aquatic crustaceans *Stenasellus virei*, *Niphargus rhenorhodanensis* and *N. virei*, which have to cope with hypoxic conditions several months per year during the hydrological cycle, displayed a high survival time in extreme hypoxia (Hervant and Mathieu, 1995; Malard and Hervant, 1998). Compared with epigean crustaceans, the most significant adaptive characteristics of these species appear to be (1) the ability to decrease their metabolic rate during hypoxia by reducing levels of activity and ventilation, and (2) the high levels of stored glycogen and arginine, making the fuelling of anaerobic metabolism possible for longer and extending the survival of these organisms (Hervant and Mathieu, 1995; Hervant et al., 1995, 1996, 1997, 1998).

Information available on crustacean metabolism during recovery from lack of oxygen is very limited. Controversy persists concerning the fate of lactate, the main anaerobic end product. Some authors have claimed that there was no (Zebe, 1982, 1991) or negligible (Hill et al., 1991b) elimination into the surrounding milieu, whilst the observations of others (Van Aardt, 1988; Hervant et al., 1995, 1996, 1997) led to the opposite conclusion. During recovery, excretion into the medium is a major mechanism for lactate disposal in epigean crustaceans such as the hypoxia-intolerant *Asellus aquaticus* and *Gammarus fossarum*, whereas hypoxia-tolerant hypogean crustaceans such as *Stenasellus virei*, *Niphargus virei* and *N. rhenorhodanensis* synthesize glycogen from accumulated anaerobic end products. This metabolite sparing is functionally advantageous, avoiding the loss of energy-rich carbon skeletons (Hervant et al., 1995, 1996, 1997).

The exact intermediary pathways involved in post-hypoxic or post-exercise lactate metabolism and their relative importance are still a matter of controversy: oxidation *versus* glycogen synthesis, the sites of gluconeogenesis and glyconeogenesis (for reviews, see Ellington, 1983; Lallier and Walsh, 1992). The occurrence of gluconeogenesis and glyconeogenesis has been demonstrated by the use of [^{14}C]lactate incorporation into several crustaceans, most notably into decapods (Phillips et al., 1977; Gäde et al., 1986; Van Aardt, 1988; Hill et al., 1991b; Lallier and Walsh, 1992; Oliveira and Da Silva, 1997). There is no information indicating whether glyconeogenesis proceeds from extracellular glucose (Cori cycle) or from the intracellular production of glucose 6-phosphate. The first pathway involves the conversion of extracellular glucose (derived from the diet or from gluconeogenesis) to glycogen. The second pathway of glycogen resynthesis during recovery is the intramuscular pathway described in vertebrates (Batty and Wardle, 1979; Moon, 1988; Pagliassotti and Donovan, 1990; Pagnotta and Milligan, 1991; Moyes et al., 1992; Palmer and Fournier, 1997). In this metabolic pathway, muscular lactate is directly transformed into glycogen inside the cell (white fast-twitch type IIb fibres; Pagliassotti and Donovan, 1990). This pathway is cytosolic because glycosyl residues derived from 2-[^{14}C]lactate are labelled only on carbons 2 and 5 without randomisation (Shiota et al., 1984).

In crustaceans, the enzymes of gluconeogenesis have never been completely characterized (for a review, see Henry et al., 1994). Phillips et al. (1977) detected very low levels of phosphoenolpyruvate carboxykinase and therefore rejected the occurrence of gluconeogenesis in the hepatopancreas. Conversely, Oliveira and Da Silva (1997) found significant levels of phosphoenolpyruvate carboxykinase in the midgut gland of a semiterrestrial crab, *Chasmagnathus granulata*. Measurable activities of gluconeogenic enzymes in the muscle, hepatopancreas and gills have been detected by Lallier and Walsh (1991). These same authors (Lallier and Walsh, 1992), using isolated hepatopancreas cells of *Callinectes sapidus*, estimated that this organ could account for only approximately 1% of post-exercise lactate clearance.

In most studies, lactate oxidation has been shown to occur simultaneously with gluconeogenesis (Phillips et al., 1977; Gäde et al., 1986; Van Aardt, 1988; Hill et al., 1991a; Lallier and Walsh, 1992). This oxidative pathway has been estimated to metabolize 10% of the injected lactate dose within 5 h in a resting river crab, *Potamonautes warreni*, but only 4% in the post-anoxic state (Van Aardt, 1988).

The aim of this study was to quantify glucose turnover rates and glycogenesis from glucose and simultaneously to determine the glyco- or gluconeogenic and oxidative capability from L-[U-^{14}C]lactate during recovery from severe hypoxia in the subterranean crustacean *Niphargus virei*.

Materials and methods

Animals

The subterranean gammarid amphipod *Niphargus virei* Chevreux, although distributed throughout Europe, is restricted to karstic aquifers. In cave biotopes, this organism digs shelters linked to the surface by very narrow tunnels within the clay substratum (Ginet, 1960). Male *N. virei* (fresh mass 128.5±4.1 mg, N=60) were collected using a net placed at the emergence of a karst system at Gueux, near Dijon, France. This karst system undergoes an annual rhythm of submersion and draining during which the oxygen partial pressure (P_{O_2}) of the water varies from normoxia (P_{O_2}=1.1 kPa) to severe hypoxia (P_{O_2}<0.09 kPa) (Hervant and Mathieu, 1995). Individuals were maintained in recirculating aquaria containing ground water together with clay and stones removed from the collection site. *N. virei* were fed with minced meat every 2 weeks. Aquaria were kept in the dark, and the temperature was maintained at 11±0.2 °C.

Experimental conditions, injection of radiolabelled compounds and recovery

Initially, animals were kept for 24 h in glass incubation flasks containing 500 ml of oxygen-saturated (normoxic, P_{O_2}=1.1 kPa) or severely hypoxic (P_{O_2}<0.03 kPa) water, as described previously (Hervant et al., 1995). Subsequently, the fate of lactate and glucose during recovery from severe hypoxia was investigated by injecting 0.5 μCi of D-[6-^{3}H]glucose (specific activity 30 mCi μmol^{-1}; 1 Ci=3×10^{10} Bq) and 0.1 μCi of L-[U-^{14}C]lactate (Na^+ salt, specific activity 0.1 mCi μmol^{-1}) (Amersham) into all individuals.

To allow comparison with post-hypoxic animals (H group), characterized by a nonsteady-state metabolism, low glycogen stores, low pH and a high lactate level, two normoxic models were produced: the N group, with steady-state metabolism, normal glycogen levels and low lactate levels, and the NLL group (normoxic animals with a lactate load) with initially normal glycogen levels and high lactate levels in disequilibrium. We were unable to produce a model with low glycogen levels since an excessive starvation period was necessary to induce lowering of glycogen stores. The NLL group was injected simultaneously with radiolabelled and unlabelled lactate to obtain a lactate load similar to that

measured in *N. virei* after 24 h of hypoxia (approximately 100 μmol lactate g^{-1} dry mass; Hervant et al., 1996).

Radiolabelled substrates were neutralized and diluted in physiological saline (pH 7.6; Hill, 1989) to give a final injection volume of 1 μl. To reduce the loss of radioactive material into the water, injections were made directly into the pericardium, through the arthrodial membrane between two dorsal segments, using a special ultrafine glass needle fixed on a 10 μl Hamilton syringe. After the injection, animals were gently placed into 500 ml sealed glass flasks containing 50 ml of normoxic water and a suspended vial containing filter paper soaked with 0.5 ml of ethanolamine/ethyleneglycol monomethylether (1:2 v/v).

After 4, 8, 16 or 24 h of recovery, individuals of all treatment groups were rapidly removed from the normoxic water, blotted dry, frozen in liquid nitrogen, lyophilised (Virtis lyophilisator, Trivac D4B) and weighed. The incubation water was acidified with 5 ml of 6.3 mol l^{-1} HCl under low pressure to release $^{14}CO_2$. The $^{14}CO_2$ released was trapped for 24 h after acid treatment. The vial containing the filter paper was removed, and 8 ml of 100 % ethanol and 10 ml of scintillation fluid (Packard Instagel, USA) were added to it. All radioactive counting was carried out using a β-counter (Packard TriCarb C300), and quenching was corrected using the external standard ratio. The efficiency of this protocol had been tested previously using a known amount of $NaH^{14}CO_3$ and was found to be between 100 and 104 %. In addition, a sample of acidified water (4 ml) was collected to measure the tritium loss from D-[6-^{3}H]glucose and the levels of non-volatile ^{14}C compounds derived from the labelled lactate in the incubation water.

Fractionation of extracts and metabolite assays

Lyophilised animals were homogenized in 1.6 ml of 0.33 mol l^{-1} ice-cold perchloric acid (PCA) using an Ultraturrax tissue grinder. Homogenates were mixed with 10 ml of chloroform/methanol (1:1 v/v). The apparatus and all glass surfaces were rinsed with 5 ml of chloroform/methanol. After 2 h at 4 °C, the extract was filtered through a fibreglass filter disc under light vacuum and the volume was corrected for evaporation (filtrate). Proteins and glycogen were retained on the filter, while the soluble fraction (glucose, lactate, amino acids and lipids) were collected in the filtrate.

Proteins and glycogen levels

The filter (see above) was desiccated and dissolved in KOH (1 mol l^{-1}) for 3 h at 80 °C, and the resulting solution was centrifuged (15 min at 2500 ***g***, 4 °C). Proteins from a sample of the supernatant were then precipitated using ice-cold PCA (0.6 mol l^{-1}, 3 h at 4 °C). Following centrifugation (15 min at 2500 ***g***, 4 °C), the protein pellet was rinsed twice with PCA (0.3 mol l^{-1}) and desiccated. After dissolution in NaOH (1 mol l^{-1}), a sample was used for protein assay according to Lowry et al. (1951). The remaining NaOH extract was used to determine the protein radioactivity. Glycogen in the PCA supernatant was precipitated by 67 % ethanol (12 h at 4 °C) and centrifuged (20 min at 2500 ***g***, 4 °C), and the pellet was rinsed twice using 67 % ethanol. After desiccation, the pellet was dissolved in 0.05 mol l^{-1} NaF (pH 4.0). A fraction of this solution was used for glycogen assay, using a standard enzymatic method (Bergmeyer, 1985), whilst the remaining fraction was used for counting the double-labelled glycogen.

Levels of glucose, lactate, amino acids and lipids

Glucose and other polar metabolites were separated from lipids by adding 4 ml of H_2O to the filtrate (see above). A sample (6 ml) of the chloroformic phase (lower phase) was washed twice with methanol/water (66:44 v/v). The solution was evaporated in a Buchi Rotovapor followed by exposure to a strong vacuum (1.5 Pa). Lipids were methylated using 0.5 mol l^{-1} HCl in methanol as described by Brichon (1984). Methylated fatty acids were extracted three times using hexane, and the radioactivity was counted. Labelled glycerol and other polar moieties of phospholipids were counted separately in the methanol/HCl fraction. The acid aqueous upper phase containing polar material (glucose, lactate and amino acids) was fractionated using a combination of precipitation and ion-exchange methods. A fraction of this upper polar phase (10 ml) was partially neutralized (pH 4.0) with ice-cold K_2CO_3 (5 mol l^{-1}) and stored for 2 h on ice. After centrifugation ($KClO_4$ precipitation; 10 min at 2000 ***g***, 4 °C), 8 ml of the supernatant was neutralized to pH 7.0, then passed through two columns of ion-exchange resins stacked in tandem: Dowex 50X8, 100–200 mesh (H^+ form) and Dowex 1X8, 100–200 mesh (acetate form), according to Rognstadt (1982). The two columns were then rinsed with 9 ml of distilled water to yield uncharged molecules, mainly glucose and glycerol. Amino acids were eluted from the Dowex 50 column using 9 ml of NH_4OH (2 mol l^{-1}), and the radioactivity was counted. Lactate was eluted from the Dowex 1 column using 10 ml of acetic acid (2 mol l^{-1}), and the radioactivity was counted. No significant radioactivity was found following elution with 2 mol l^{-1} formic acid (no pyruvate present). Glucose and lactate were assayed spectrophotometrically (Beckman DU-6) in the different eluates using standard enzymatic methods (Bergmeyer, 1985). The effectiveness of the fractionation procedures and of the ion-exchange chromatography was tested by using a known mixture of pure glycogen, glucose, lactate and amino acids. Recovery of these metabolites ranged from 91 % (amino acids and glucose) to 97 % (lactate). These efficiencies were used for corrections in subsequent calculations.

Statistics and calculations

Glucose (R_G) and lactate (R_L) turnover rates were calculated using classical treatments of single-pool systems (Radziuk et al., 1978; Garin et al., 1987; Wolfe, 1992). These variables were only quantified when glucose or lactate levels were constant during the experiments. The rate of oxygen consumption ($\dot{M}_{O_2}$) was measured as described previously (Hervant et al., 1998), and CO_2 specific activity was estimated using the ratio of CO_2 radioactivity to $\dot{M}_{O_2}$, assuming arbitrarily a respiratory quotient (RQ) of 1. The rate of synthesis of metabolites from labelled

Table 1. *Changes in levels of metabolites in* Niphargus virei *during normoxia (N), normoxia after a lactate load (NLL) or recovery from severe hypoxia (H)*

Time (h)	[Glycogen] (μmol glycosyl g^{-1} dry mass)			[Glucose] (μmol g^{-1} dry mass)			[Lactate] (μmol g^{-1} dry mass)		
	N	NLL	H	N	NLL	H	N	NLL	H
0	242±16	245±16	147±20^{a,b}	6.7±0.2	6.6±0.3	7.3±0.5	8.8±0.8	105.5±9.0^{a}	108.4±9.1^{a}
4	267±19	247±19	148±19^{a,b}	8.9±0.2^{t}	6.9±0.2^{a}	7.2±0.2^{a}	9.2±0.9	84.1±6.5^{a}	67.7±6.0^{a,t}
8	248±15	267±19	178±13^{a,b}	7.2±0.3	6.5±0.2	7.5±0.2^{b}	8.3±0.7	64.0±5.9^{a,t}	51.0±4.7^{a,b,t}
15	255±20	280±23	193±26^{b}	7.1±0.1	6.5±0.2	7.1±0.2^{b}	9.6±0.8	46.2±3.8^{a,t}	35.5±3^{a,b,t}
24	253±17	300.3±19.0^{t}	207±13^{a,b,t}	6.6±0.2	7.0±0.2	6.7±0.1	9.0±0.7	20.3±1.5^{a,t}	11.8±1.8^{b,t}

All values are expressed as mean ± S.E.M. (N=9–10).

^{a}Significantly different from the N group; ^{b}significantly different from the NLL group; ^{t}significantly different from the value at time 0 (P<0.05).

substrates was quantified using the ratio A_i/A_t, where A_i is the area under the curve of radioactivity incorporated into glycogen and A_t is the area under the curve of tracer specific activity (Katz, 1979). This calculation integrating for time was preferred to the simple ratio of [final accumulated product radioactivity]/[precursor specific activity] used by others (Johnson and Bagby, 1988; Gleeson and Dalessio, 1990; Pagnotta and Milligan, 1991). The use of this methodology to calculate the rate of glycogen synthesis from glucose gave reasonably consistent results throughout the experiments in all groups and was therefore considered reliable.

The same methodology was used to estimate incorporation of ^{14}C from lactate into glycogen, glucose and CO_2 (see Table 6 for equations). A 15 h recovery period was chosen to avoid the low specific activities of the precursor lactate observed at 24 h.

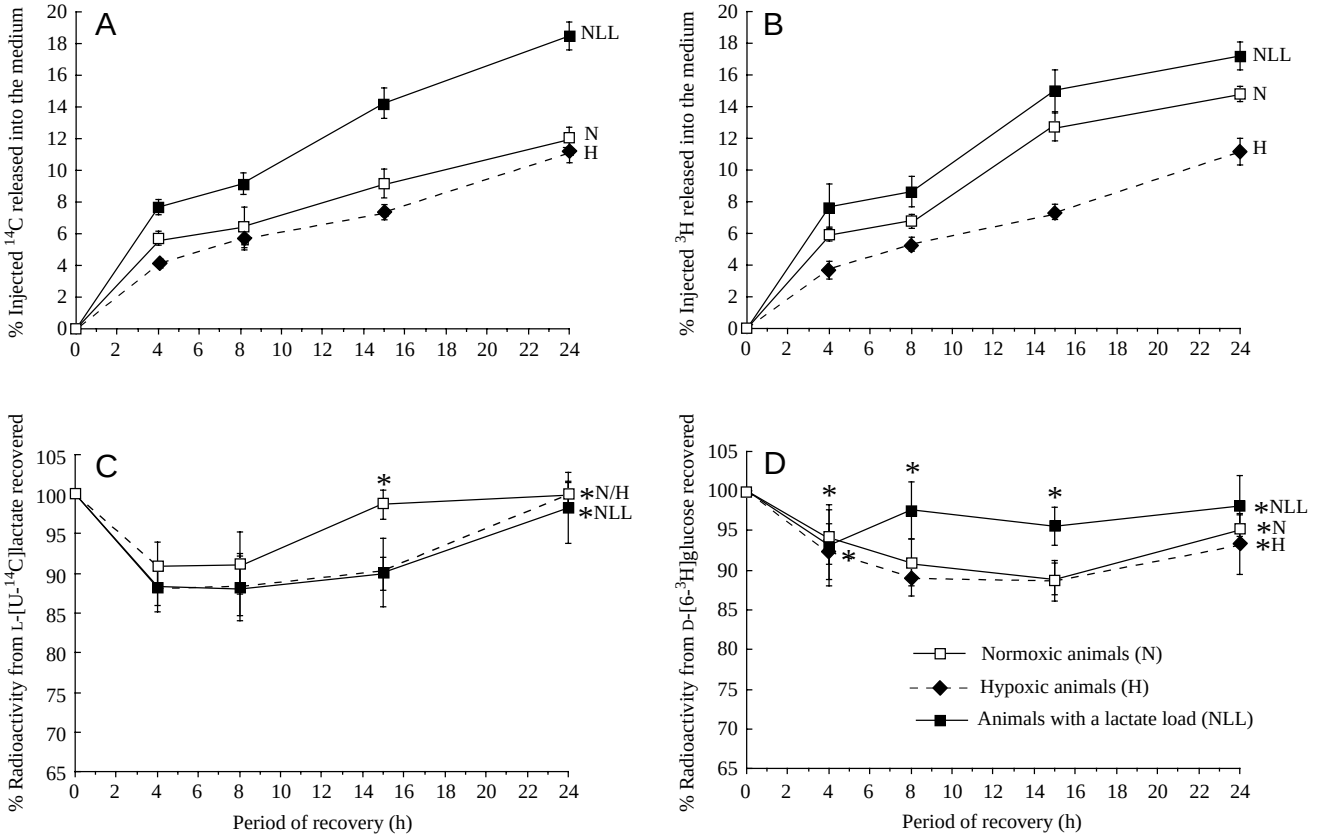

Fig. 1. (A,B) Percentage of injected radioactivity derived from [^{14}C]lactate (A) or [6-^{3}H]glucose (B) in the subterranean crustacean *Niphargus virei* found in the external medium after acidification. (C,D) Percentage of injected radioactivity derived from [^{14}C]lactate (C) or [6-^{3}H]glucose (D) recovered in the analysed metabolites and compartments. All values are means ± S.E.M. for N=9–10 animals. An asterisk (*) indicates that values are not significantly different (P>0.05) from the control value (t=0 h). N, normoxia; NLL, normoxia after a lactate load; H, recovery from severe hypoxia.

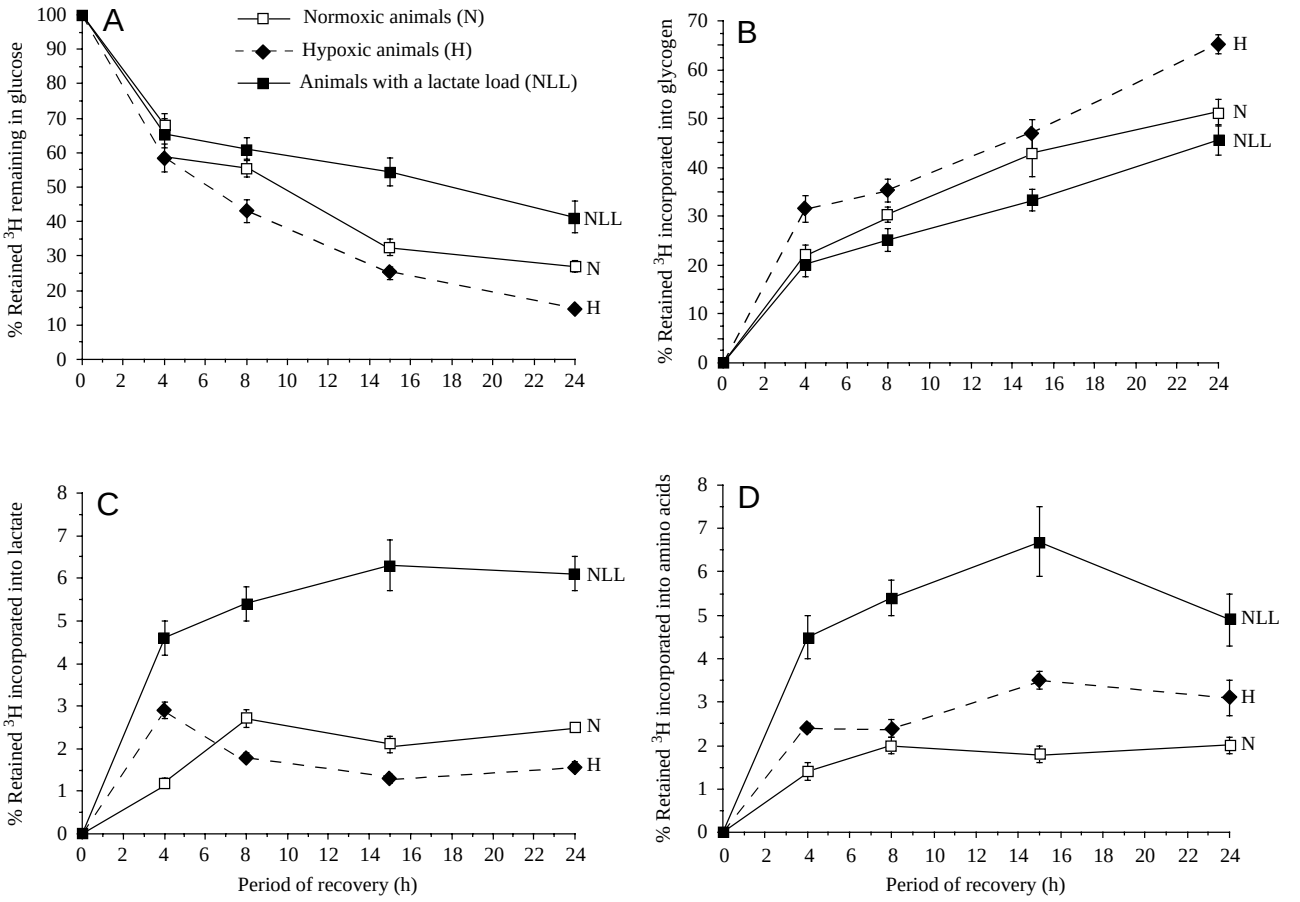

Fig. 2. (A) Time course of utilization of radioactivity from D-[6-^{3}H]glucose. (B,C,D) Time course of incorporation of radioactivity derived from D-[6-^{3}H]glucose into glycogen (B), lactate (C) and amino acids (D) in the subterranean crustacean *Niphargus virei*. Values are expressed as a percentage of the injected radioactivity retained in the tissues. All values are means ± S.E.M. for N=9–10 animals. At all times of recovery, values are significantly different (P<0.05) from the control value (t=0 h). N, normoxia; NLL, normoxia after a lactate load; H, recovery from severe hypoxia.

The contribution of glucose to glycogen synthesis was estimated in similar manner to that used by Johnson and Bagby (1988) using the ratio ($SA_{G(n)}$×100)/(mean SA_{Glc}), where $SA_{G(n)}$ is the specific activity of the synthesized [^{3}H]glycogen during the experiments and SA_{Glc} is the specific activity of free [^{3}H]glucose. A value near 100 suggests that glycogen comes from the direct uptake of free glucose and phosphorylation, while a value close to zero indicates that glycogen is synthesized from other sources.

All comparisons between the H, NLL and N groups were made using Student's t-tests. Values are presented as means ± S.E.M.

Table 2. *Specific activity of [^{3}H]glucose and [^{3}H]glycogen in* Niphargus virei *after* D-*[6-^{3}H]glucose injection during normoxia (N), normoxia after a lactate load (NLL) or recovery from severe hypoxia (H)*

Time after [6-^{3}H]glucose injection (h)	Glucose specific activity (disints min^{-1} µmol^{-1})			Glycogen specific activity (disints min^{-1} µmol^{-1})		
	N	NLL	H	N	NLL	H
4	98469±4215	94914±5968	81110±6410[a]	945±18	772±13[a]	2317±19[a,b]
8	77237±3674	94090±5591[a]	57405±4447[a,b]	1277±11	964±10[a]	2653±10[a,b]
15	46158±2935	83788±6512[a]	35111±2118[a,b]	1797±15	1214±13[a]	3264±24[a,b]
24	42047±1533	58913±4989[a]	21979±1260[a,b]	1971±9	1514±9[a]	3187±9[a,b]

All values are expressed as means ± S.E.M. (N=9–10).
Specific activities are expressed as disints min^{-1} µmol^{-1} glucose equivalent for a dose of 10^6 disints min^{-1} 1 g dry mass.
[a]Significantly different from the N group; [b]significantly different from the NLL group (P<0.05).

Results

Metabolite content

Body glucose levels of *Niphargus virei* remained constant during recovery from hypoxia (Table 1). No consistent significant effects of injection, hypoxia or lactate load were observed on glucose level in all three groups. Conversely, glycogen levels were stable in N animals, but net synthesis occurred in NLL and H animals (2.28 and 2.48 μmol glycosyl h^{-1} g^{-1} dry mass, respectively). Initial lactate concentrations, as expected, did not differ in NLL and H animals (P<0.05; Table 1). During recovery from hypoxia, lactate levels decreased in NLL and H animals, reaching the normoxic value after 24 h in H animals. The rates of net lactate utilization were not significantly different in H and NLL *N. virei* (3.55±0.31 and 4.03±0.30 μmol lactate h^{-1} g^{-1} dry mass, respectively). Indeed, these values were closer still when energy expenditure was taken into account (U_{LI}, see below and Table 3).

Table 3. *Variables of glucose and lactate metabolism in* Niphargus virei *during normoxia (N), and during recovery from severe hypoxia (H) during normoxia after a lactate load (NLL)*

	N	NLL	H
$\dot{M}_{O_2}$ (μmol O_2 h^{-1} g^{-1} dry mass)	13.5±0.9	17.2±2.2	21.9±1.7[a]
R_G (μmol glucose h^{-1} g^{-1} dry mass)	0.405	0.216	0.65
R_{GI}	18.4	7.5	17.8
R_L (μmol lactate h^{-1} g^{-1} dry mass)	1.12	–	–
U_L (μmol lactate h^{-1} g^{-1} dry mass)	–	3.55±0.31	4.03±0.30
R_{LI}	24.8	–	–
U_{LI}	–	61.9	55.2

Values are expressed as means ± S.E.M. (N=9–10).

$\dot{M}_{O_2}$, rate of oxygen consumption = $\frac{\int_0^{24} M_{O_2} dt}{24}$ (from Hervant et al., 1998), where t is time.

R_G, [6-^{3}H]glucose turnover rate. The correlation (r) between the experimental data and the single-exponential curve was 0.956 in N animals, 0.939 in NLL and 0.997 in H animals.

R_{GI} , glucose turnover index = $R_G \times 100 \times$ number of carbon atoms in the molecule $\times \dot{M}_{O_2}^{-1}$ (Garin et al., 1987); this adjustment for energy expenditure was necessary when the rate of oxygen consumption was not constant (Henderson et al., 1986).

R_L, L-[U^{14}C]lactate turnover rate. Since the lactate pool was in equilibrium in N animals, lactate turnover was calculated using lactate specific activity and single-exponential curves plotted against time.

U_L, net lactate utilization rate. Since the lactate pool was not in equilibrium in the NLL and H groups, lactate utilization was calculated from the difference in lactate content between 0 and 24 h.

R_{LI}, lactate turnover index, or U_{LI}, net lactate utilization index = (R_L or U_L) × 100 × number of carbon atoms in the molecule × $\dot{M}_{O_2}^{-1}$.

[a]Significantly different from the N group.

Radiotracer analysis

Radioactivity recovery

Fig. 1 presents the percentage recovery of the injected radioactivity in the various metabolites analyzed 4, 8, 15 and 24 h after injection. Progressive leakage of non-volatile radioactivity into the medium was observed from the [^{14}C]lactate as well as the [^{3}H]glucose data (Fig. 1A,B). This leakage was greatest in NLL and lowest in H animals. These differences imply that leakage was therefore mainly due to metabolic processes and not to loss from the injection site. The sum of the ^{14}C radioactivities recovered in the different metabolites analyzed was lower than 100 % 4 h after injection of lactate but approximately 100 % after 15 h in N animals and after 24 h in H and NLL animals (Fig. 1C). Recovery of [^{3}H]glucose (Fig. 1D) was significantly higher in NLL (approximately 98 % from 8 to 24 h) than in N or H animals (approximately 93 % at 24 h) (P<0.05).

Fate of D-[6-^{3}H]glucose

Fig. 2 shows the incorporation of D-[6-^{3}H]glucose into the different metabolic fractions. The NLL group showed the lowest glucose utilization rate (significantly different between groups; P<0.05), with only 59 % removal at 24 h compared with 73 % and 85 % in N and H animals, respectively (Fig. 2A). ^{3}H incorporation into glycogen was higher in H (65.2±1.8 %) than in N (52.2±2.8 %) and NLL (45.6±3.2 %) animals (Fig. 2B) (P<0.05). The NLL group had the highest proportion of ^{3}H activity incorporated into molecules resulting from glycolysis (i.e. lactate 6.1±0.4 %; amino acids 4.9±0.6 %) (Fig. 2C,D) and tritiated water released into the medium (Fig. 1B). Conversely, the highest [^{3}H]glucose utilization rate was in H animals (Fig. 2A), which had the highest incorporation into glycogen (65.2±1.8 %), the lowest incorporation into lactate (1.6±0.1 %, Fig. 2C) and an intermediate level of incorporation into amino acids (3.1±0.4 %, Fig. 2D; significantly different between groups, P<0.05).

A semi-logarithmic decay plot of the glucose specific activity data given in Table 2 allowed glucose turnover rate (R_G, Table 3) to be calculated for the three groups. R_G was larger in H than in N animals and was lowest in NLL animals (significantly different between groups, P<0.05). However, since the rate of O_2 consumption was enhanced during post-hypoxic recovery (Hervant et al., 1998), R_G was scaled relative to $\dot{M}_{O_2}$ (Garin et al., 1987) to give the glucose turnover index (R_{GI}, Table 3). R_{GI} was similar in the N and H groups but was 2–3 times lower in the NLL group. Specific activities of tritiated glycogen (Table 2) were significantly higher in H than in N and NLL animals at all times post-injection (P<0.05). Consequently, the rate of glycogenesis calculated from incorporation of [^{3}H]glucose into glycogen was

significantly enhanced in H with respect to N and NLL animals, whereas no significant differences in net rates of cold glycogen synthesis were apparent between any group (Table 4, $P>0.05$). From the glucose/glycogen synthesis ratio of Johnston and Bagby (1988), it was observed that the percentage of glycogen synthesized directly from [^{3}H]glucose was enhanced during recovery from hypoxia relative to NLL animals (Table 4).

Fate of L-[U-^{14}C]lactate

Fig. 3 shows the rate of incorporation of L-[U-^{14}C]lactate into different metabolic fractions during the 24 h recovery period. The NLL group showed the lowest lactate utilization rate, with a removal of 69.5 % in 15 h, compared with 84.4 % and 94.6 % for the N and H groups, respectively (Fig. 3A). In H animals, the ^{14}C label was initially incorporated into glucose (16 % at 4 h) (Fig. 3C) and later into glycogen (53 % at 15 h

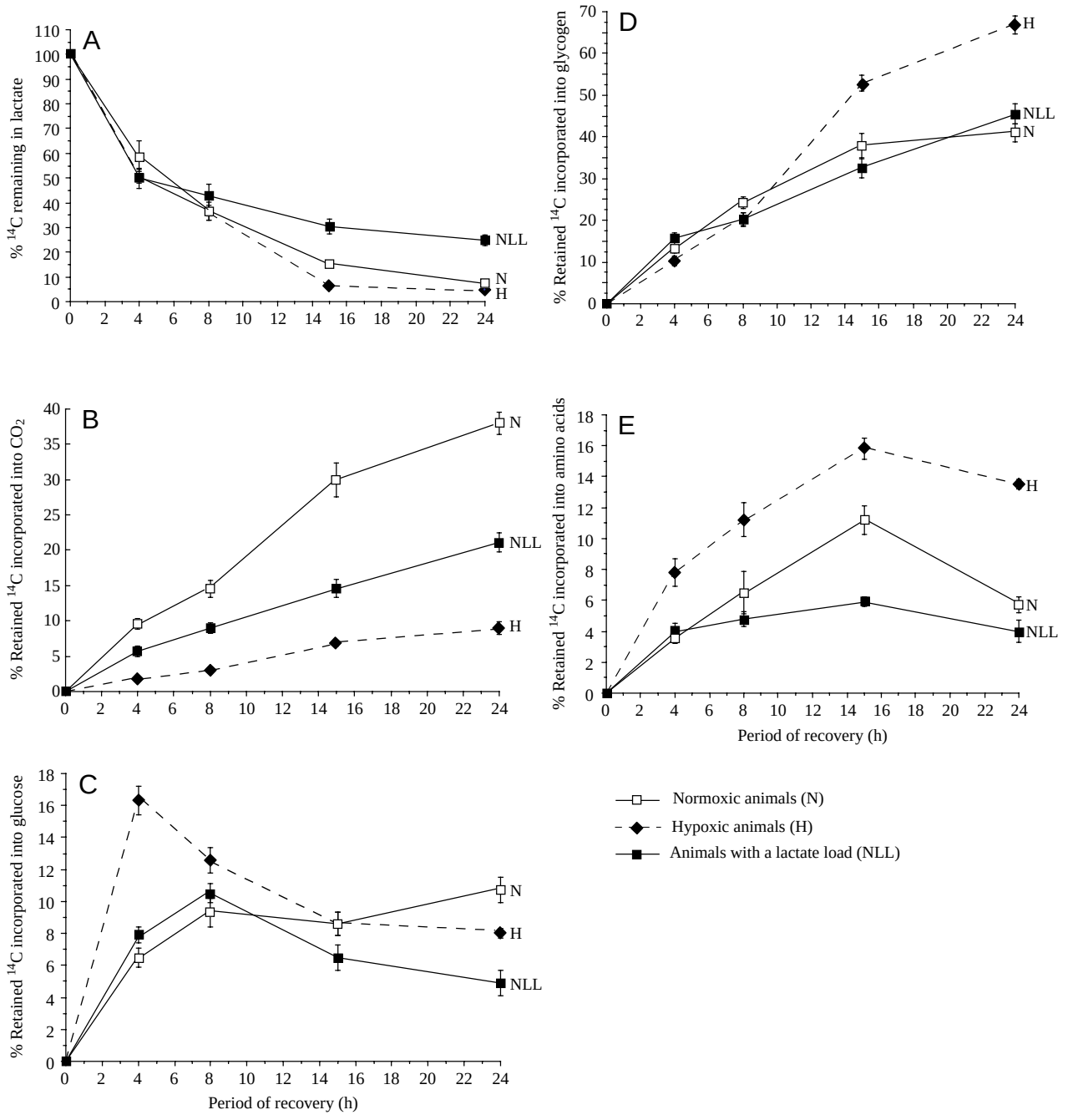

Fig. 3. (A) Time course of utilization of radioactivity from L-[U-^{14}C]lactate. (B–E) Time course of incorporation of radioactivity derived from L-[U-^{14}C]lactate into CO_2 (B), glucose (C), glycogen (D) and amino acids (E) in the subterranean crustacean *Niphargus virei*. Values are expressed as a percentage of the injected radioactivity retained in the tissues. All values are means ± S.E.M. for N=9–10 animals. At all times of recovery, values are significantly different ($P<0.05$) from the control value (t=0 h). N, normoxia; NLL, normoxia after lactate load; H, recovery from severe hypoxia.

Table 4. *Rate of glycogenesis from D-[6-^{3}H]glucose in* Niphargus virei *during normoxia (N), normoxia after a lactate load (NLL) or recovery from severe hypoxia (H)*

	N	NLL	H
Glycogenesis from [^{3}H]glucose (μmol glucosyl h^{-1} g^{-1} dry mass)	0.273±0.040	0.243±0.024	0.660±0.067[a,b]
Net glycogen synthesis (μmol glucosyl h^{-1} g^{-1} dry mass)	0.44±1.30	2.3±1.4	2.48±1.3
% Direct synthesis of glycogen from glucose	–	11.5±0.5	24.7±1.5[b]

Values are means ± S.E.M. (N=9–10).

Glycogenesis from [^{3}H]glucose; μmol of [^{3}H]glucose incorporated into glycogen = $\frac{\int_0^{24} A_{G(n)}dt}{\int_0^{24} SA_{Glc}dt}$, where A is activity (disints min^{-1}), t is time (min), G(n) is glycogen and SA_{Glc} is the specific activity of glucose (disints min^{-1} μmol^{-1}).

Net glycogen synthesis = $\frac{[G(n)]_{24} - [G(n)]_0}{24}$.

% Direct synthesis = $\frac{\Delta SA_{G(n)}}{\int_0^{24} SA_{Glc}dt} \times 100$.

[a]Significantly different from the N group; [b]significantly different from the NLL group (P<0.05).

and 65 % at 24 h) (Fig. 3D). Incorporation into amino acids was higher in H (15.8±0.7 % at 15 h) than in N (11.2±0.9 %) or NLL (5.9±0.3 %) animals (Fig. 3E). Conversely, incorporation into CO_2 was considerably lower in H (6.8±0.4 % at 15 h) than in NLL (14.6±1.3 %) or N (30.0±2.4 %) animals (significantly different between groups, P<0.05) (Fig. 3B).

Specific activities of lactate, glucose, glycogen and CO_2 (expressed here as disints min^{-1} μatom^{-1} carbon) are presented in Table 5. CO_2 specific activity was calculated from $\dot{M}_{O_2}$ using an RQ of 1. The lactate pool (see Table 1) was approximately 10 times smaller in N than in H and NLL animals. Thus, the initial lactate specific activity will be approximately 10 times higher in N animals than in the other groups. Lactate specific activity decreased with time in the H and N groups, whilst it did not change significantly with time from 4 h to 15 h in NLL animals. Lactate specific activity was identical in N and NLL animals at 15 h. In all three groups, incorporation into glucose was maximal as early as 4 or 8 h post-injection, followed by a progressive decrease in glucose specific activity with time. Glycogen specific activity increased with time in all groups, to the greatest extent between 8 and 15 h. Glucose and glycogen specific activities were of the same order of magnitude in the three groups, even though initial lactate specific activity was approximately 10 times higher in N animals. Conversely, CO_2 specific activity was significantly larger in N than in NLL (approximately 2.4-fold) and H (approximately 7.6-fold) animals.

Lactate turnover rate (R_L) is presented in Table 3 for the N group. In NLL and H animals, net lactate utilization was estimated from the decrease in lactate concentration per unit body mass *versus* time since lactate concentration varied with time. Incorporation of label derived from lactate (Table 6) was almost identical for glycogen, glucose and CO_2 in N animals. The lactate load in NLL animals resulted in a 2.6-fold increased incorporation of label into glycogen relative to N animals. The more complex metabolic pattern of post-hypoxic recovery (H animals) resulted in significantly enhanced incorporation of [^{14}C]lactate into glycogen (12.6-fold), glucose, (8.2-fold) and, to a lesser extent, CO_2 (2.4-fold). In H animals, incorporation into CO_2 was only approximately 20 % of that into glycogen and 36 % of that into glucose.

Incorporation of ^{3}H and ^{14}C into polar and non-polar lipid fractions was negligible, indicating that no synthesis or exchange process with lipids was occurring in any of the groups. Despite incorporation of ^{14}C into amino acids being greater in H than in N or NLL animals (Fig. 3E; significantly different between groups, P<0.05), there was no significant incorporation into proteins in any of the groups (P>0.05).

Injection of [^{14}C]lactate resulted in the appearance of label in the non-acid-labile fraction of the medium. This radioactivity reached 11 % (at 24 h) of the injected dose in H animals and 18 % in NLL animals. These results were not analysed further in the present study.

Discussion

Glucose metabolism and glucogenesis

Detailed laboratory experiments recently conducted on three stygobitic crustaceans, as well as previous laboratory studies,

Table 5. *Carbon specific activity in lactate, glucose, glycogen and CO_2 after L-[U-^{14}C]lactate injection in* Niphargus virei *during normoxia (N), normoxia after a lactate load (NLL) or recovery from severe hypoxia (H)*

Time after [^{14}C]lactate injection (h)	Lactate specific activity (disints min^{-1} µatom^{-1} C)			Glucose specific activity (disints min^{-1} µatom^{-1} C)		
	N	NLL	H	N	NLL	H
4	6439±328	1330±217^{a}	660±49^{a,b}	414±37	279±18^{a}	527±42^{b}
8	4458±349^{t}	1436±183^{a}	569±38^{a,b}	298±28^{t}	435±39^{a,t}	376±24^{a,b,t}
15	1582±112^{t}	1369±421	158±9^{a,b,t}	296±27^{t}	260±34	285±23^{t}
24	767±29^{t}	2009±277^{a}	77±19^{a,b,t}	228±24^{t}	167±26^{t}	291±5^{a,b,t}

Time after [^{14}C]lactate injection (h)	Glycogen specific activity (disints min^{-1} µatom^{-1} C)			CO_2 specific activity (disints min^{-1} µatom^{-1} C)		
	N	NLL	H	N	NLL	H
4	90±2	113±3^{a}	120±1^{a}	2096±146	879±104^{a}	275±104^{a,b}
8	168±1^{t}	132±2^{a,t}	194±3^{a,b,t}	1592±136^{t}	686±58^{a}	240±20^{a,b}
15	250±2^{t}	201±3^{a,t}	572±6^{a,b,t}	1635±131^{t}	554±50^{a,t}	258±4^{a,b}
24	269±2^{t}	263±2^{t}	544±4^{a,b,t}	1373±59^{t}	538±33^{a,t}	198±28^{a,b}

All values are expressed as means ± S.E.M. (N=9–10).

^{a}Significantly different from the N group; ^{b}significantly different from the NLL group; ^{t}significantly different from the value at time 0 (P<0.05).

showed that groundwater macro-crustaceans were much more resistant to hypoxia than were epigean-dwelling species (for a review, see Malard and Hervant, 1998).

Previous studies have shown that, during post-hypoxic recovery, the subterranean crustaceans *Stenasellus virei*, *N. virei* and *N. rhenorhodanensis* displayed a faster restoration of glycogen, phosphagen and ATP levels to pre-hypoxic values than did epigean species (*Gammarus fossarum* and *Asellus aquaticus*). Moreover, during recovery, the main fate of accumulated end products (mainly lactate, with alanine and succinate produced in small quantities) was not excretion into the medium but anabolism. A good stoichiometric relationship between glycogen resynthesis and removal of accumulated end products was observed for *Stenasellus virei* and both *Niphargus* species, leading to the hypothesis that these subterranean species have a relatively high capacity for glyconeogenesis (Hervant et al., 1995, 1996, 1997).

The present experiments were undertaken first to verify the

Table 6. *Incorporation of L-[U-^{14}C]lactate into glycogen, glucose and CO_2 in* Niphargus virei *during normoxia (N), normoxia after a lactate load(NLL) or recovery from severe hypoxia (over a 15 h period)*

	N			NLL			H		
	G(n)	*Glc*	CO_2	*G(n)*	*Glc*	CO_2	*G(n)*	*Glc*	CO_2
Lactate incorporated (µatoms h^{-1} g^{-1} dry mass)	5.24±0.75	4.86±1.41	6.08±0.47	13.7±4.0^{a}	2.93±0.87	9.17±2.47	66.1±13.5^{a,b}	39.8±3.0^{a,b}	14.3±2.7^{a}

G(n), glycogen; Glc, glucose.

µatoms of lactate incorporated into glucose, glycogen or CO_2 = $\frac{\int_0^{15} A_{\text{Product}}\,dt}{\int_0^{15} SA_{\text{Lactate}}\,dt}$, where A_{Product} is activity (disints min^{-1}) in glucose, glycogen or CO_2, SA_{lactate} is the specific activity of lactate (disints µatom^{1}) and t is time (h).

Glycogen and CO_2 were considered to accumulate during the experiment.

Glucose was considered to be exchanged with other metabolites: $A_{\text{Glc}} = \int_0^{15} A_{\text{Glc}}dt \times R_{\text{G}}$, where R_{G} is [6-^{3}H]glucose turnover rate (see Table 3).

Values are means ± S.E.M. (N=9–10).

^{a}Significantly different from the N group; ^{b}significantly different from the NLL group (P<0.05).

hypothesis that glycogen is synthesised *de novo* from lactate and/or glucose. Second, the simultaneous study, on the one hand, of glucose metabolism and its incorporation into glycogen and, on the other hand, of lactate incorporation into glucose and glycogen permitted insight into both glycogen synthesis pathways: the intracellular route without free glucose formation, and the exchange of lactate with gluconeogenic cells leading to extracellular glucose synthesis. Finally, the experiment to measure the recovery of label in CO_2 was designed to explore the energy source utilized by *N. virei* during post-hypoxic recovery.

Because of the small size (apprimately 100 mg) of *N. virei*, the present study was conducted on the whole animal; hence, no analysis of inter-organ exchange could be achieved between muscle and a hypothetical gluconeogenic organ (liver equivalent). In the absence of clear experimental data on crustaceans, gluconeogenesis was predicted to operate following the classical pathway including pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose diphosphatase and glucose-6-phosphatase. In the following discussion, glycogen is considered to be intracellular, glucose to be mainly extracellular (in the haemolymph) and lactate can be exchanged between the intra- and extracellular compartments.

Glucose utilization and glycogen synthesis

D-[6-^{3}H]glucose is considered to be an irreversible glucose tracer. Radioactivity from injected D-[6-^{3}H]glucose is lost during exchanges in the Krebs cycle (Wolfe, 1990) and is excluded from gluconeogenesis. Consequently, detritiation of D-[6-^{3}H]glucose can be interpreted as glucose catabolism in the whole animal. The fate of D-[6-^{3}H]glucose is relevant to several processes. (i) In non-gluconeogenic or non-glyconeogenic tissues, D-[6-^{3}H]glucose enters two perfect isotopic sinks: glycogen and pyruvate, the label finally being lost in the Krebs cycle. (ii) In gluconeogenic tissues, several imperfect sinks exist. In these cells, containing glucose-6-phosphatase, recycling between glucose, glucose 6-phosphate and glycogen occurs without tritium loss; label is lost in the mitochondria between pyruvate and phosphoenolpyruvate and, consequently, there is no recycling from [^{3}H]lactate or pyruvate into glucose or glycogen. (iii) In the muscular glyconeogenic pathway, no tritium is lost by reversing pyruvate kinase, so ^{3}H-labelled lactate (synthesized from glucose) does not leave the cell and is therefore used (without tritium loss) for restoration of glycogen stores (Wolfe, 1990). Thus, the detritiation of glucose is accomplished by mitochondrial processes, namely glucose oxidation and/or gluconeogenesis, but not by glyconeogenesis.

Loss of tritium during post-hypoxic recovery after 15 h was only 50 % of that in NLL animals and 59 % of that in N animals (Fig. 2). This result and our findings for [^{14}C]lactate provide evidence for the existence of an enhancement of gluconeogenesis in H animals. During recovery from hypoxia, glucose oxidation was depressed by more than 50 %. This inhibition of glucose oxidation appeared to be dependent on a physiological regulation mechanism other than lactate load, since detritiation was enhanced in NLL animals.

As previously observed, glucose content remained remarkably stable in spite of the high levels of stress induced by hypoxia, lactate load and/or injection (Table 1). This apparent stability masked an enhancement of D-[6-^{3}H]glucose turnover rate in the order H>N>NLL (Table 3). The glucose turnover rate (R_G) measured in this study in N animals (equivalent to 1.35 μmol glucose min^{-1} kg^{-1} wet mass; Table 3) was of the same order of magnitude as R_G values reported for other poikilotherms such as fish (0.3–13 μmol min^{-1} kg^{-1} wet mass: Garin et al., 1987; Machado et al., 1989; Weber and Haman, 1996) and lizards (0.46 μmol min^{-1} kg^{-1} wet mass at 15 °C and 1.04 μmol min^{-1} kg^{-1} wet mass at 25 °C; Guppy et al., 1987).

During post-hypoxic recovery (H group), R_G was enhanced (three times that of NLL animals and 1.6 times that of N animals). However, this measure of cellular glucose entry using R_G is not a truly valid comparison, since the overall energy expenditure was inflated by the repayment of the O_2 debt during recovery (Hervant and Mathieu, 1995; Hervant et al., 1998). A better comparison is obtained by taking metabolic rate ($\dot{M}_{O_2}$) into account (Henderson et al., 1986) using the glucose turnover index (R_{GI}; Garin et al., 1987) (Table 3). The R_{GI} incorporates the percentage participation of glucose in overall energy expenditure if glucose is assumed to be entirely oxidized. Experimental R_{GI} values found in the subterranean *N. virei* (7.5 in NLL and 18.4 in N animals) are similar to R_{GI} values calculated for poikilotherms such as fish (3.9–30; for reviews, see Garin et al., 1987; Machado et al., 1989) and lizards (20.6 at 15 °C, 15.3 at 25 °C and 9.9 at 35 °C; Guppy et al., 1987). In the present study, R_{GI} values underwent no significant modification in H animals compared with the N group. In contrast, the NLL group (characterized by a high lactate load and high glycogen levels) showed a large decrease in the rate of entry of glucose into metabolism.

After entry of glucose into the cell, glycogenesis represents an important fate of glucose, especially during recovery from hypoxia. In the present study, the rate of glycogenesis was estimated using the turnover rates of radioactive isotopes. This general methodology is open to criticism since it introduces confusion between net molecular flow and radioisotopic exchanges. In many pathways, high levels of isotopic exchanges or recycling result in errors in the measured rates of appearance of molecules (Katz, 1979). When applied to the rate of net glycogen synthesis, our calculations (see Table 4) gave very similar results for all three groups. After correction for $\dot{M}_{O_2}$, the glycogen turnover index (calculated in a manner similar to R_{GI}, see Table 3) for NLL animals was 8.5 and for H animals was 17.8, showing that the lactate load in the NLL group reduced both the rate of glucose entry and the incorporation of glucose into glycogen.

The glucose:glycogen synthesis ratio (Johnson and Bagby, 1988; Table 4) estimated after 24 h during net glycogen synthesis was very low in NLL animals (12 %) and

approximately twice as high (25 %) in H animals. These values are much lower than those calculated for higher vertebrates, suggesting that the subterranean crustacean *N. virei* glycogenesis utilizes extracellular glucose poorly, particularly after lactate loading (NLL animals), and instead relies on glyconeogenic intracellular processes. At the same time, the reduced tritium loss into the medium in post-hypoxic animals relative to the other groups (Fig. 1B) indicates that glucose was poorly oxidized after entering the cell and was mainly directed towards glycogen synthesis. In the normoxic group, where metabolism was at equilibrium, glycogenesis from glucose represented 65 % of glucose turnover. Other fates for glucose were oxidation and distribution among different pools such as lactate, glycerol and/or amino acids.

Gluconeogenesis, glyconeogenesis and oxidation of L-[U-^{14}C]lactate

As was the case for 6-[^{3}H]glucose, the recovery of radioactive label in the different fractions reached 100 % 24 h after injection (Fig. 1C). Between 4 and 15 h, the missing radioactivity (0–11.5 %) was probably temporarily incorporated into glycolytic intermediates and into the citric acid cycle (Hill et al., 1991a).

A major process occurring during recovery from anaerobiosis or exercise in crustaceans is the clearance of end products, mainly lactate. Ellington (1983) indicated that anaerobic end products can be disposed of by three possible methods: complete oxidation, conversion back into storage products such as glycogen, and/or excretion. Previous studies have found no evidence of substantial excretion of lactate during recovery in *N. virei* (Hervant et al., 1996).

It has been claimed that, in numerous epigean and hypogean crustaceans (Phillips et al., 1977; Ellington, 1983; Albert and Ellington, 1985; Taylor and Spicer, 1987; Hill et al., 1991a; Anderson et al., 1994; Hervant et al., 1995, 1996, 1997), removal of lactate from the tissues or haemolymph is slow during recovery from anoxia or severe hypoxia, and that these organisms do not have particularly efficient systems for metabolizing this end product. Nevertheless, the present study showed that the hypogean *N. virei* has an apparent total lactate (extracellular and intracellular) turnover rate (R_L) (Table 3) similar to values reported in other ectotherms: the R_L measured here (1.1 μmol lactate h^{-1} g^{-1} dry mass at 11 °C, equivalent to 3.7 μmol lactate min^{-1} kg^{-1} wet mass) is of the same magnitude as in the rainbow trout *Oncorhynchus mykiss* (R_L=2.8 μmol lactate min^{-1} kg^{-1} at 9.5 °C; Dunn and Hochachka, 1987) and higher than observed in the coho salmon *Oncorhynchus kisutsch* (R_L=1.3 μmol lactate min^{-1} kg^{-1}; Milligan and McDonald, 1988), starry flounder *Platichthys stellatus* (R_L=0.8 μmol lactate min^{-1} kg^{-1}; Milligan and McDonald, 1988), catfish *Ictalurus punctatus* (R_L=2.25 μmol lactate min^{-1} kg^{-1} at 25 °C; Cameron and Cech, 1989) and various lizard species (R_L=0.06 μmol lactate min^{-1} kg^{-1}; Guppy et al., 1987), but remains lower than that estimated in rat (R_L=57 μmol lactate min^{-1} kg^{-1} at 38 °C; Fréminet and Poyart, 1975; the R_L value obtained in *N. virei* would be approximately 35 μmol lactate min^{-1} kg^{-1} at 38 °C using a Q_{10} of 2.3).

In *N. virei*, the labelled lactate was incorporated mainly into glycogen, CO_2, glucose and amino acids. This distribution of label is very different from that reported in trout *Oncorhynchus mykiss* after exercise (Milligan and Girard, 1993), in which significant incorporation into proteins and lipids was observed. The absence of such an incorporation into proteins and lipids in the present study suggests the dominance of catabolism of these macromolecules.

Incorporation of ^{14}C from lactate into amino acids in all three groups of subterranean *N. virei* was considerably lower than that observed in the marine crabs *Carcinus maenas* (Hill et al., 1991a) and *Menippe mercenaria* (Gäde et al., 1986), in which 70 % of the [^{14}C]lactate appeared in the amino acid fraction during both normoxia and recovery from anaerobiosis. In *N. virei*, the incorporation of ^{14}C into amino acids (Fig. 3E) was greater in post-hypoxic (15.5 % at 15 h) than in normoxic (10.9 %) animals, which suggests an increase in transaminations linked to increased gluconeogenesis and/or to enhanced deamination of AMP to IMP, although no modification of NH_4 excretion occurs (Hervant et al., 1996). The most important labelled amino acid is probably alanine (Gäde et al., 1986), since it has been implicated in the metabolic response to severe hypoxia (Hervant et al., 1995, 1996).

Despite the limitations of this method of assessing lactate metabolism, as outlined by several authors (Katz, 1979; Wolfe, 1990), incorporation of [^{14}C]lactate into CO_2, glucose and glycogen could be estimated (Table 6). The calculation used overcomes the difficulty arising from the difference in initial lactate specific activity in the different groups. In normoxic animals, levels of incorporation of [^{14}C]lactate into glycogen, glucose and CO_2 were approximately the same. In NLL animals, the initial lactate load resulted in an enhancement of incorporation into glycogen, without significant modification of gluconeogenesis or oxidation. The post-hypoxic recovery state induced, relative to NLL animals, a considerable simultaneous increase in gluconeogenesis (14-fold) and glycogenesis (fivefold) with no significant modification of lactate oxidation. The mean ratio of gluconeogenesis + glyconeogenesis to oxidation was approximately 6.5 times higher in H than in N animals and three times higher in the H than in the NLL group.

The present attempt to quantify fluxes from ^{14}C-labelled lactate can be compared with rates of molecular synthesis as determined from unlabelled measures, e.g. from rates of glycogen synthesis (Table 4) and $\dot{M}_{O_2}$ (Table 3) or from single-pool analysis (R_G, Table 3). Molecular synthesis of glucose or glycogen-glucosyl is usually considered to represent one-sixth of the total carbon incorporated (Gleeson and Dalessio, 1990). This estimate gives the rates of synthesis of glycogen and glucose as 0.87 and 0.81 μmol h^{-1} g^{-1} dry mass, respectively, in N animals, as 2.3 and 0.48 μmol h^{-1} g^{-1} dry mass in NLL animals and as 11.0 and 6.6 μmol h^{-1} g^{-1} dry mass in H animals. Assuming a RQ of 1, oxidation of lactate represented

45 % of $\dot{M}_{O_2}$ in N, 53 % in NLL and 65 % in H animals. In contrast, in H animals, glycogen and glucose synthesis estimated using this method are much greater than net glycogen synthesis determined from glucose-glycogen synthesis (600 %, Table 4) and glucose turnover rate (980 %, Table 3). Moreover, the high level of incorporation into CO_2 found in H animals is inconsistent with the low proportion of the injected dose found in the acid-labile fraction (Fig. 3). This excessive synthesis in post-hypoxic animals could result from a massive recycling of labelled material in this group, leading to a dilution of [^{14}C]lactate and to an enrichment of products, namely glycogen, glucose and CO_2.

We can conclude that lactate was approximately equally distributed between the oxidative pathway, glucose synthesis and glycogen synthesis in N animals. A lactate load induced muscular glyconeogenesis. During recovery from severe hypoxia, glyconeogenesis and gluconeogenesis were enhanced at the expense of the oxidative pathway. More precise quantitative estimates of these changes will require the determination of ^{14}C recycling rates.

The present study confirmed the existence of gluconeogenesis in crustaceans, as demonstrated previously at the organismal level in epigean crustaceans, most notably in decapods (Phillips et al., 1977; Gäde et al., 1986; van Aardt, 1988; Hill et al., 1991a). In the hypogean *N. virei*, incorporation of ^{14}C from lactate into glucose occurred much faster (as soon as 4 h post-injection) than in epigean-dwelling species (Stetten, 1982; Van Aardt, 1988; Hill et al., 1991a; Henry et al., 1994). This rapid incorporation of label derived from lactate into glucose, largely preceding a massive incorporation into glycogen, seems to be consistent with a quantitatively important involvement of the Cori cycle. However, estimates of glycogenesis rates from glucose incorporation (Table 4) indicated that the utilization of this pathway was low, representing no more that 10–25 % of net glycogen turnover. This estimate was reinforced by a comparison of the ratio of ^{14}C to ^{3}H in glycogen and glucose, which quantifies the relative participation of [^{14}C]lactate and [^{3}H]glucose in glycogenesis. This ratio was relatively constant throughout the duration of an experiment within each group, but differed between the three groups. The ^{14}C:^{3}H ratio was 26.2±2.8 times higher for glycogen than for glucose in the N group. In the NLL group, it was 48.9±6.6 times higher, indicating that a greater proportion of the synthesized glycogen was derived from [^{14}C]lactate. Conversely, it was lower in the H group (13.6±2.3-fold), further indicating enhanced participation of the Cori cycle (relative to glycogenesis) during post-hypoxic recovery. This pattern was in accordance with that observed using the method of Johnson and Bagby (1988) (Table 4). A ^{14}C:^{3}H ratio that was considerably higher for glycogen than for glucose indicated that glycogen was largely synthesized from lactate in the cell, without dilution of the ^{14}C label by other ‘cold’ contributors (amino acids and/or glycerol), or exchange at the mitochondrial level. During post-hypoxic recovery (H animals), entry of circulating glucose into the tissues was enhanced, as shown by a higher R_G, a higher rate of glycogenesis from glucose, and a lower ^{14}C:^{3}H ratio in glycogen in comparison with normoxic animals.

The present data suggest that there may be two distinct pathways of glycogen synthesis from lactate. The bulk of the lactate was probably transformed to glycogen inside muscle cells without any marked isotopic dilution by the reverse action of pyruvate kinase. This mechanism was favoured by high lactate levels. The second, normally minor, gluconeogenic pathway, which is enhanced during recovery from hypoxia, probably produced extracellular glucose through several mitochondrial steps and therefore resulted in the dilution of labelled lactate (for a review, see Milligan and Girard, 1993).

During recovery from exercise, the energy status of the white muscle of the trout *Oncorhynchus mykiss* recovered faster than did its ATP concentration, as a consequence of the high level of activity of AMP deaminase and of the very high ratio of ATP to free ADP concentration (Dobson et al., 1987; Moyes et al., 1992; Schulte et al., 1992). This very high ratio of ATP to free ADP was shown to inhibit mitochondrial respiration, but to favour the reversal of pyruvate kinase. A similar pattern could occur in anoxia-tolerant subterranean crustaceans since the present study found a reduced proportion of oxidized lactate in H animals (Fig. 3), implying inhibition of pyruvate dehydrogenase. *In vitro* studies have shown that even low levels of fatty acids strongly inhibit this enzyme (Moyes et al., 1992). It is possible that the reduced levels of lactate oxidation and glucose metabolism, associated with the high $\dot{M}_{O_2}$ observed in post-hypoxic *N. virei* (Hervant et al., 1998), result from enhanced fatty acid oxidation. In another hypogean crustacean of similar ecology, glycerol levels were enhanced twofold during recovery fom hypoxia (Hervant et al., 1997), indicating high levels of lipolysis.

In humans, fatty acids are the main substrate oxidized during recovery from intense exercise, despite high levels of lactate (Bangso et al., 1991). This pattern of regulation of muscular glyconeogenesis observed in vertebrates (Ryan and Radziuk, 1995) could also explain the patterns described in the present study in the anoxia-resistant hypogean crustacean *N. virei*. Clearly, the high lactate level was not the only factor inducing a post-hypoxic metabolic response, but rather acted synergistically with other variables such as intracellular lactate localization (Ryan and Radziuk, 1995), low pH (Bonen et al., 1990), a high level of free inorganic phosphate and a high ATP to free ADP ratio (Schulte et al., 1992).

In conclusion, the metabolic response of *N. virei* during post-hypoxic recovery essentially exhibited the characteristics of mammalian white muscle (Dobson et al., 1987; Moyes et al., 1992; Schulte et al., 1992). Restoration of glycogen levels occurred at a similar rate in post-hypoxic animals (H), which had low initial glycogen levels, and in normoxic animals (NLL), which had the same initial lactate content as H animals but a normal initial glycogen concentration. The newly synthesised glycogen originated mainly from lactate, since synthesis from lactate (as opposed to glucose) was seven times faster in H and 17 times faster in NLL than in N animals. The use of the glucose oxidation pathway was diminished during

recovery from hypoxia, but no significant modifications occurred in lactate-loaded animals.

The bulk of glycogen synthesis occurred by an intracellular pathway without glucose synthesis. Both gluconeogenesis and glyconeogenesis were considerably enhanced during post-hypoxia recovery. To a certain extent, glyconeogenesis (but not gluconeogenesis) was also enhanced in the NLL group. Oxidative metabolism of lactate was apparently depressed in post-hypoxic (H) hypogean animals and to a lesser extent in the NLL group.

The incorporation of radioactivity from ^{14}C-labelled lactate was slower into glycogen than into glucose, but remained high 24 h post-injection (Fig. 3C,D). Since glucose is mainly an extracellular metabolite, this observation is consistent with the hypothesis of two distinct sites for glycogen restoration in hypogean crustaceans: a gluconeogenic organ (a liver equivalent) and a glyconeogenic organ (a muscle equivalent).

There was no evidence of large-scale protein utilization for energy metabolism during recovery, since no significant increase in NH_4^+/NH_3 excretion was observed in a previous study during post-hypoxic recovery (Hervant et al., 1996). Enhanced incorporation of label into amino acids could, however, be an indication of enhanced transamination and/or of an active purine cycle during recovery. Since carbohydrates are actively resynthesized and oxidized more slowly during the payment of the O_2 debt, it seems clear that respiration was augmented by an enhancement of lipid catabolism.

This research was supported by the French Ministry of Education, France, grant 93-1154, and by funds from the University Claude Bernard-Lyon I and the National Centre of French Scientific Research (CNRS). The authors thank Dr Tristam Eldershaw (University of Tasmania, Hobart, Australia) for his valuable assistance in correcting parts of the English manuscript. They are also pleased to acknowledge the constructive criticisms of the anonymous referees.

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