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FLEXIBILITY OF ANAEROBIC METABOLISM IN AQUATIC OLIGOCHAETES (*TUBIFEX* SP.). BIOCHEMICAL AND CALORIMETRIC CHANGES INDUCED BY A DEPROTEINIZED HYDROLYSATE OF BOVINE BLOOD

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Abstract—1. Anaerobic processes were studied in *Tubifex* and other aquatic invertebrates. After the aerobic–anoxic transition of *Tubifex*, succinate accumulated up to about 25 μmol/g W_d within the first hour of anoxia, but steady-state levels were established after 4 hr at only 10 μmol/g W_d in an open-flow system.

2. Proprionate accumulated after a lag of 30 min and reached steady-state concentrations of about 30 μmol/g W_d after 5 hr of anoxia.

3. Lactate concentrations did not increase above 4 μmol/g W_d under anoxia. Its accumulation was not induced by exposure to a blood extract, although *Tubifex* has the potential for lactate production.

4. The initial rates of glycolytic endproduct accumulation were increased in the presence of the deproteinized blood extract by 60% (succinate, 0–30 min anoxia) and by 50% and 90% (proprionate, 30–60 min anoxia; 1% and 2% blood extract, respectively). The maximum and steady state *levels* of these metabolites were not influenced by the hydrolysate of blood.

5. Aerobic and anaerobic heat dissipation of *Lumbriculus variegatus* was stimulated by the addition to the medium of a deproteinized hydrolysate of bovine blood. Oxygen uptake of *Cyclops abyssorum* increased similarly upon the addition of the blood extract under hypoxia.

INTRODUCTION

Evidence has accumulated over many years that anaerobic metabolism in invertebrates is highly flexible. (1) Interspecifically, the ratio of total metabolism under anoxic and aerobic conditions usually varies between 10% and 40% as measured by direct calorimetry (total heat dissipation; reviewed by Gnaiger, 1983a,b). (2) Intraspecifically, this anoxic/aerobic ratio is a function of animal size (Pamatmat, 1980). Total metabolic rate under environmental anoxia is not a constant for a given species but varies according to temperature and physiological state (Gnaiger, 1980; Shick, 1981). Furthermore, anoxic metabolism is influenced by pharmaceutical and toxicological agents (Gnaiger, 1981, 1983a). (3) Most spectacularly, anaerobic metabolism is involved in locomotory burst activity and in periods of high metabolic demand during recovery (physiological hypoxia; reviewed by Livingstone, 1982; Ellington, 1983; De Zwaan and Putzer, 1985). (4) Different anaerobic pathways are switched on during either physiological or environmental anoxia even within one species (Schöttler, 1979; Zebe *et al.*, 1981; Putzer, 1984). Lactate or opines (Gäde, 1983) are the endproducts of glycolytic pathways during burst activity with high rates of ATP-turnover, whereas the succinate–propionate–acetate pathways supply metabolic energy under environmental anoxia with high efficiencies but at low rates (Gnaiger, 1983b). (5) Anoxic acclimation is accompanied by a typical shift from initial endproduct accumulation (alanine, succinate) to long-term endproduct excretion (propionate, acetate) (reviewed by De Zwaan, 1983). The flexibility of anaerobic metabolism offers a broad potential for assessing the mechanism of action of pharmaceuticals or toxicants on cellular systems. Even the mere test of the effect of drugs on aerobic and anaerobic processes provides a distinction between specific influences on the respiratory chain as opposed to general effects on catabolism or anabolism (Gnaiger, 1981, 1983a). In this paper we describe such tests which provide insights into the comparative physiology of anaerobiosis and may at the same time serve as a valuable method in the pharmaceutical and in the environmental sciences. Deproteinized blood and tissue extracts are therapeutically applied to support recovery of patients after surgery or intoxication and to enhance healing of problematic wounds such as burns, ulcerations and decubitus. In an attempt to characterize the specific effect and to describe the active agent, Jaeger *et al.* (1965) reported a 30–80% increase of respiration of liver homogenates in the presence of a low molecular fraction of blood. The increased respiration is not due to decoupling of electron transport phosphorylation (Schäfer and Lamprecht, 1965), but the mechanism of action remains unknown. Other data (Riede *et al.*, 1975; Wolff, 1975) suggest that the effects are not restricted to regulatory aspects of catabolism but that anabolic capacity (as evidenced by cell proliferation)

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Table 1. Glycolytic endproducts in 100% GSA-40 solution

	Endproduct (μmol dm^{-3})*
L-Lactate	34.69 ± 2.89 (3)
Succinate	3.61 ± 1.44 (3)
Propionate	1.40 ± 0.48 (3)
Acetate	31.21 ± 3.28 (3)

*Mean ± standard deviation; number of determinations in brackets.

is increased by these extracts. Preliminary experiments with a deproteinized hydrolysate of bovine blood (GSA-40) showed that this extract is compatible with most commercially available preparations, using the increase of mitochondrial respiration as a measure of activity (Knapp, 1983; unpubl.). We studied the effect of GSA-40 on aquatic invertebrates which are tolerant to anoxic conditions. The regulatory effects of the blood extract on glycolytic flux were tested by determinations of glycolytic endproducts and by direct calorimetric and respirometric measurements in open-flow systems.

MATERIALS AND METHODS

Animals

Benthic oligochaetes (*Tubifex* sp.; *Lumbriculus variegatus*) were purchased from a local aquarium supply firm. *L. variegatus* was sorted out for microcalorimetric experiments. The remaining Tubificidae (predominantly *Tubifex tubifex*) were used in biochemical tests. They were acclimated in aerated water at 20°C for at least one week without the addition of food. Prior to experiments the animals were cleaned and weighed (wet weight, W_w; dry weight = $0.17 \times W_w$). An additional respirometric experiment was performed with crustacean plankton (*Cyclops abyssorum*) obtained from Kalbelesee (Hochtannberg, Vorarlberg, Austria). *C. abyssorum* was acclimated to the experimental temperature (6°C) in the laboratory.

The deproteinized blood extract

GSA (Tradename Seractiv®, manufactured by GEBRO, Fieberbrunn, Austria) is an extract of whole bovine blood treated with proteases and deproteinized by alcohol precipitation and ultrafiltration ($\leq$10,000 dalton). The salt content was reduced chromatographically. The original GSA-40 solution contained approximately 2–4 mg ml^{-1} free amino acids and a similar amount of peptides. Some metabolites are also listed in Table 1. Balance to 40 mg ml^{-1} results mainly from salts and sugars.

GSA-40 and the synthetic test solution containing all low molecular weight substances as determined in original GSA-40 were supplied by GEBRO (G. Broschek K.G., Fieberbrunn, Austria).

The original (100%) solutions were diluted to 1 or 2 vol% with tap water for incubation experiments with the animals.

Aerobic and anoxic exposure

All experiments were performed in open-flow systems. For exposure to aerated water (aerobic) or water equilibrated with pure nitrogen (anoxic) the tubificids (300–400 mg W_w) were enclosed in 4.7 ml glass chambers at a constant perfusion rate ranging from 30 to 40 ml hr^{-1}. All connections were made of stainless steel to avoid oxygen diffusion. Figure 1 clarifies the experimental regime of aerobic and anoxic tests with GSA-40 solutions and the respective controls.

Biochemical determinations

After sampling, animals were washed in tap water to remove the adhering surface film of GSA-40 solution. Cleaned animals were immediately dropped into 4 ml of 6% perchloric acid, homogenized with an Ultraturrax and centrifuged at 15,000 *g* for 30 min. After centrifugation, neutralization with $KHCO_3$, lactic acid was determined enzymatically (Bergmeyer, 1974). Succinate was determined after esterification with methanol–sulfuric acid by gas–liquid chromatography using 2.5 mM maleic acid (added to the PCA extract) as internal standard. Volatile fatty acids were isolated from perchloric acid extracts by steam distillation and determined by gas–liquid chromatography on a Varian 3700 Aerograph. For all GLC analyses a 10% SP-1000/1%H_3PO_4 on 100/120 Chromosorb W AW column was used (2 mm i.d. × 12′).

Respirometric and microcalorimetric experiments

The respirometric and direct calorimetric methods were described in detail elsewhere. The CYCLOBIOS Twin-Flow Microrespirometer was used with 40 individuals of *C. abyssorum* (1.5 mg total dry weight, W_d) in a 0.5 ml animal chamber at a flow rate of 5.7 ml hr^{-1} (Gnaiger, 1983c). A LKB-2107 flow-sorption microcalorimeter was adapted to operate under anoxic conditions (Gnaiger, 1980; 1983a,c). Ten individuals of *L. variegatus* (about 17 mg W_d) were perfused in the 0.5 ml animal chamber with water equilibrated with nitrogen or air at a flow rate of 15 ml hr^{-1}.

RESULTS

Anoxic metabolism: glycolytic endproducts

GSA-40 solution contains considerable amounts of those chemicals which predominate as glycolytic endproducts in euryoxic animals (Table 1). These high background concentrations rendered the

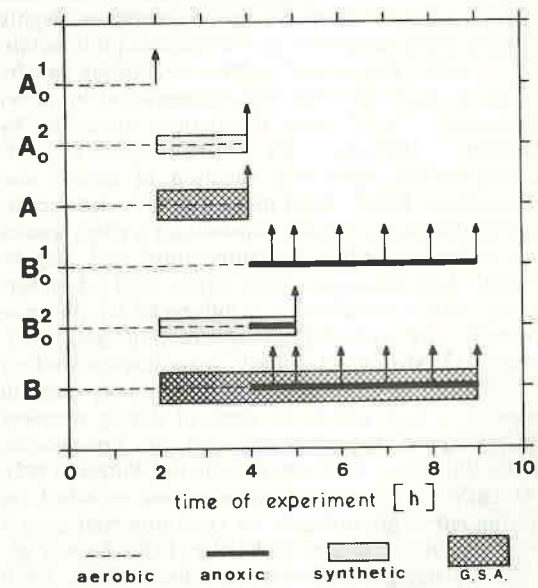

Fig. 1. Experimental regime for investigating the glycolytic endproduct accumulation under aerobic (A_0^1, A_0^2, A) and anoxic (B_0^1, B_0^2, B) conditions. The controls (1) were determined in pure tap water (A_0^1, B_0^1). The dotted area shows the period of exposure to the "synthetic control solution" (A_0^2, B_0^2). The hatched area shows the period of exposure to the GSA-40 solution (A, B). The arrows indicate the times of sampling and fixation of the animals.

Table 2. Effect of GSA-40 (vol%) and time of anoxic exposure (min) on glycolytic endproduct accumulation under aerobic (A) and anoxic (B) conditions

	Time (min)	Conc. (%)	Endproduct (μmol/g W_d)			
			L-Lactate	Succinate	Propionate	Acetate
A_0^1	0	0	n.d.	0.0 (5)	0.0 (5)	7.91 (1)
A_0^2	0	2	1.16 (1)	1.34 (1)	0.27 (1)	14.40 (1)
A	0	1	n.d.	1.79 ± 1.37 (5)	0.46 ± 0.25 (5)	15.51 ± 3.41 (5)
	0	2	2.80 (1)	1.82 ± 1.23 (5)	0.23 ± 0.10 (5)	10.80 ± 3.88 (5)
B_0^1	30	0	n.d.	11.59 ± 2.78 (9)	1.04 ± 0.39 (9)	8.82 ± 1.07 (9)
	60	0	3.54 ± 1.45 (4)	24.32 ± 4.75 (11)	9.91 ± 1.99 (11)	17.92 ± 1.17 (11)
B_0^2	60	2	3.99 ± 2.18 (2)	24.06 ± 2.49 (2)	10.99 ± 1.44 (2)	21.50 ± 0.14 (2)
B	30	1	n.d.	21.74 ± 1.24 (4)	3.30 ± 0.63 (4)	17.10 ± 1.53 (4)
	30	2	n.d.	19.75 ± 1.93 (5)	3.22 ± 1.59 (5)	9.99 ± 2.13 (5)
	60	1	3.80 ± 1.41 (2)	27.98 ± 2.50 (3)	16.65 ± 1.65 (3)	17.90 ± 0.47 (3)
	60	2	n.d.	24.94 ± 2.26 (4)	20.45 ± 1.10 (4)	21.50 ± 2.80 (4)

*Mean ± SD; number of determinations in brackets. The experimental values are compared to the controls in pure tap water (A_0^1, B_0^1) and to the effect of the synthetic control solution (A_0^2, B_0^2).
n.d. = not determined; W_d = dry weight.

quantification of organic acid excretion difficult. We decided to use tissue accumulation as experimental parameter. Controls with synthetic test solution (Table 1) were performed to test for transport of these substances into the tissues. Accumulation may also be due to aerobic glycolysis during the initial disturbance of the animals. Therefore the oligochaetes were acclimated to the test chamber for 2 hr. Similarly, they were exposed to the test solutions for 2 hr prior to the aerobic–anoxic transition (Fig. 1). The concentrations reached in aerobic controls (A) were subtracted from the levels of endproduct accumulation in the anoxic tests (B) for interpretation of the drug effect on anaerobic metabolism. Lactate plays a minor role in metabolism of the aquatic oligochaetes under environmental anoxia. Occasional determinations of lactate did not indicate any significant changes (Table 2).

Succinate was undetectable under aerobic control conditions (A_0^1) but increased to 0.22 μmol/g W_w (A_0^2, synthetic control solution) and 0.31 μmol/g W_w (A, GSA-40) during 2 hr aerobic exposure. In these 2% solutions the succinate concentration was 0.07 μmol/dm^3 (Table 1). Under anoxic conditions the initial rate of succinate accumulation was significantly enhanced by GSA-40 relative to the anoxic controls (Fig. 2). After 45–60 min of anoxia, succinate reached maximum concentrations irrespective of the addition of GSA-40. The concentration of this primary anoxic endproduct decreased after 2 hr of anoxia although succinate is not excreted by the oligochaetes.

Propionate, a secondary anoxic endproduct, accumulated at a rate of 18 μmol hr^{-1} (g W_d)$^{-1}$ after a lag of 30 min. Accumulation was linear up to 60 min (Fig. 2I). 1% and 2% solutions enhanced propionate accumulation rates to 27 and 35 μmol hr^{-1} (g W_d)$^{-1}$, respectively, between 30 and 60 min of anoxia. A steady-state concentration of 30 μmol/g W_d was found after 2 hr in the presence of GSA-40, but in the control this concentration was observed after 5 hr only (Fig. 2).

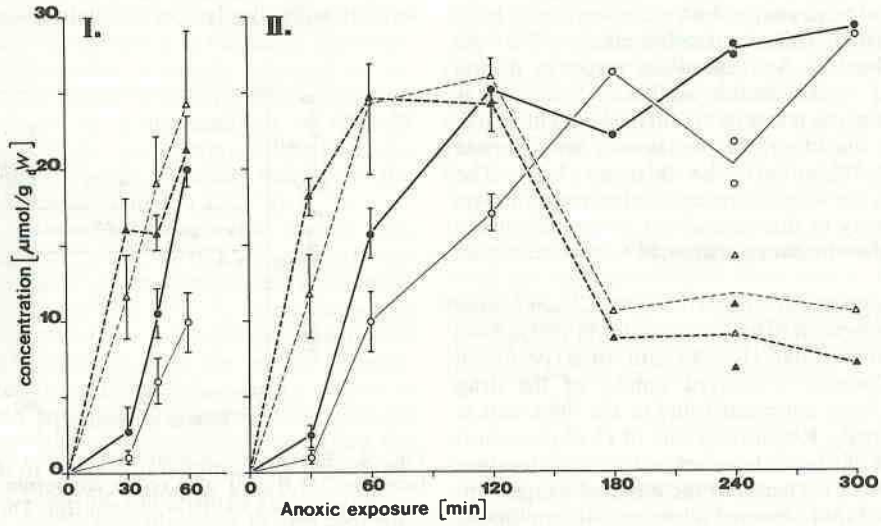

Fig. 2. Effect of GSA-40 and time of anoxic exposure on accumulation of succinate (triangles) and propionate (circles). The bars represent the SD of the means. The anoxic controls (dotted lines; B_0^1) are compared with the anoxic GSA-40 tests corrected for the aerobic accumulation of endproducts (full lines, B–A: from the concentrations in experiment B the concentrations reached in experiment A were subtracted; see Fig. 1 for explanation). I: 2% GSA-40 solution. II: 1% GSA-40 solution.

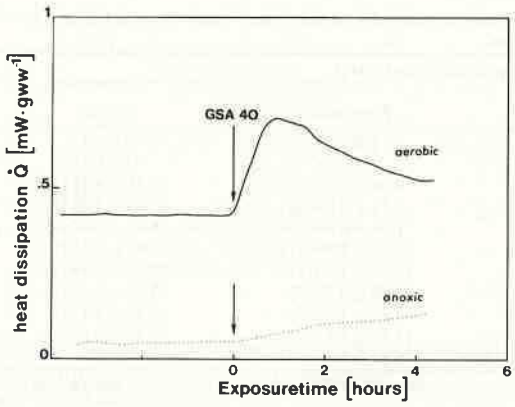

Fig. 3. Continuous calorimetric records (LKB flow-sorption microcalorimeter) showing the effect of GSA-40 (2%) on aerobic (full line) and anoxic (dotted line) rate of heat dissipation. The recorded voltage of the thermopiles is directly proportional to the power, dQ/dt. Prior to the anoxic exposure to GSA-40 solution, anoxic acclimation lasted for 8 hr. After 4 hr perfusion with GSA-40, the onset of bacterial growth interfered with the measurement.

Acetate concentrations displayed a wide scatter (Table 2) which may be partly explained by the large concentration of this endproduct in the original GSA-40 and synthetic test solution (Table 1).

Anoxic and aerobic metabolism: direct calorimetry and respirometry

Microcalorimetric experiments provide continuous records of the rate of heat dissipation representing a signal integrating over all biological, aerobic and anoxic, activities. The anoxic effect of GSA-40 on *L. variegatus* was delayed by 20 min (Fig. 3). The animals were acclimated to anoxia for 8 hr. During such a period of anoxic exposure, steady-state concentrations of glycolytic endproducts are established (Fig. 2). Hence processes other than organic acid accumulation (e.g. endproduct excretion) must have been stimulated. This calorimetric effect of GSA-40 was reproducible. An immediate response of the oligochaetes to the aerobic addition of GSA-40 is apparent from the power–time curve shown in Fig. 3. After 1 hr maximum of metabolic activity was reached at 75% above the reference level. The difference in the kinetics under aerobic versus anoxic conditions may be due to a slower anaerobic uptake of the effective substance relative to aerobic transport mechanisms.

Oxygen uptake by the arthropod *C. abyssorum* increased immediately upon the addition of GSA-40 solution to the hypoxic (10% air saturation) perfusion medium, although a delayed uptake of the drug might have been anticipated due to the thick cuticle of these animals. Respiratory rate of *C. abyssorum* is independent of oxygen level below 10% air saturation (Gnaiger, 1983c). Therefore the elevated oxygen uptake exceeded that observed under aerobic conditions and at comparable levels of starvation.

DISCUSSION

The stimulating effect of the bovine blood extract (GSA-40) on the metabolism of invertebrates from different phyla (annelids and crustaceans; Figs 3 and 4) proves that the active substance contained in this deproteinized hydrolysate is not specific for mammalian cells. Blood extracts lose their stimulating effect after ashing (Pichotka *et al.*, 1965). Similarly, synthetic solutions of inorganic and organic low-molecular weight components contained in the extracts were without effect ($9.9 \pm 2\,\mu$mol/g W_d in controls and $10.7 \pm 1.4\,\mu$mol/g W_d in 2% synthetic solution corrected for the aerobic content), whereas GSA-40 solution exerted a concentration-dependent effect on the metabolic rate of oligochaetes ($16.7 \pm 1.7\,\mu$mol/g W_d in 2% GSA-40 solution after 60 min).

Lactate and acetate appear in relatively high concentrations in the blood extract (Table 1). While the latter is apparently taken up by tubificids under aerobic conditions both from synthetic and GSA-40 solution, lactate levels remain low under these conditions (Table 2). Acetate exerts an inhibitory effect on the absorption of propionate by the polychaete *Arenicola marina* (Holst and Zebe, 1984). Despite the possibility that this is also true for oligochaetes, propionate concentrations were slightly increased after 2 hr of aerobic perfusion with either synthetic or GSA-40 solution (Table 2). Propionate is excreted under anoxia (Schöttler and Schroff, 1976) and is channeled into the citric acid cycle under aerobic conditions. This may explain the slightly increased level of succinate in the presence of synthetic and GSA-40 solution. This is not likely due to the absorption of succinate itself, which under anoxia is not excreted either (Schöttler and Schroff, 1976). Free amino acids were contained in millimolar concentrations in 100% GSA-40 and synthetic test solutions. Uptake of amino acids may be another factor influencing concentrations of citric acid cycle intermediates. The same is true for glucose contained in either solution.

To avoid interference by initial organic acid absorption with glycolytic accumulation during anoxia,

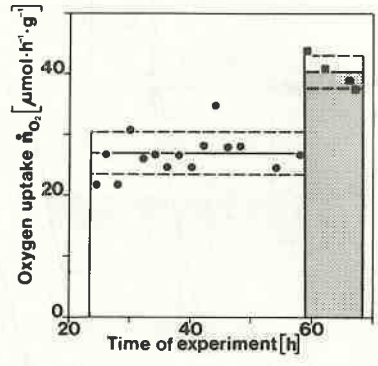

Fig. 4. Effect of GSA-40 (5%) on oxygen uptake [μmol·hr^{-1}(g W_d)$^{-1}$] of *Cyclops abyssorum* at 9.9% air saturation (p_{O_2} = 1.94 kPa = 14.5 mmHg). The symbols indicate hourly averages of the continuous record of the CYCLOBIOS Twin-Flow Microrespirometer. The horizontal lines show the mean rates (full lines) and standard deviations (broken lines) during control conditions (open area) and after addition of GSA-40 to the perfusion medium (hatched area).

anoxic conditions were initiated after a 2 hr aerobic perfusion with synthetic or GSA-40 solution (Fig. 1, B_0^2 and B). We assume that by this time steady-state conditions with respect to absorption processes were established in the openflow system, and further changes were due to catabolic rather than absorption mechanisms.

It is generally assumed that the unidentified low molecular agent contained in blood extracts exerts its therapeutic effect by stimulating respiratory rate (for references see introduction). However, the fact that aerobic as well as anaerobic energy metabolism is increased by the addition of GSA-40 solution (Figs 3 and 4) proves that the therapeutic substance does not specifically act on the respiratory enzymes. Two mechanisms or a combination of both may explain our results. (1) An insulin-like effect (Bachmann *et al.*, 1968; Parade *et al.*, 1968) may give rise to an increased mobilization of glycogen and utilization of glucose which is catabolized by aerobic or anaerobic pathways. (2) A stimulation of anabolic (biosynthetic) processes may increase ATP demand which is met under aerobic and anoxic conditions by the respective catabolic pathways. In any case, the reduced glycogen utilization and diminished lactate production under hypoxia in the presence of blood extract (Somogyi *et al.*, 1979) cannot be due to an inhibitory effect of extracts on glycolytic mechanisms, but must be due to the compensatory stimulation of oxidative metabolism.

The convergence of endproduct concentration after 5 hr (propionate) and 1 hr (succinate) (Fig. 2) must not be mistaken as indicating that the effect of GSA-40 on anaerobic metabolism of *Tubifex* is restricted to the initial period of anoxia. It is well known that these endproducts of anerobic catabolism reach steady-state levels after a transitory period, after which time propionate and acetate are continuously formed but excreted (Schöttler and Schroff, 1976; Seuß *et al.*, 1983; Putzer, 1984; 1985). The presence of these organic acids in the GSA-40 solution prevented an accurate estimation of their excretion rates under the influence of this drug. However, a continuing stimulation is clearly indicated by the microcalorimetric response of anoxically acclimated oligochaetes (8 hr) upon the addition of GSA-40 (Fig. 3). Similarly, the rate of oxygen uptake by *C. abyssorum* under hypoxia remained well above the control rate for more than 8 hr (Fig. 4).

A 1.5 and 1.9-fold increase in the rate of propionate accumulation (30–60 min of anoxia) was induced by 1% and 2% GSA-40 solution, respectively, relative to the control rate (Fig. 2). Interpretation in terms of ATP-turnover or catabolic heat dissipation is complicated, since not only the rate of accumulation but also the pathway (glycogen or malate as precursor) can be affected. Furthermore, GSA-40 solution enhanced succinate production during the first half-hour of anoxia. In the following period, the high levels of succinate inhibited its further rate of accumulation below that observed in the controls, where succinate accumulated linearly during the first hour of anoxia (Fig. 2). We conclude from this result that under the present experimental conditions succinate concentrations between 22 and 30 μmol/g W_d are the regulatory signal inhibiting its further accumulation, irrespective of the rate at which this concentration is reached and independent of the level of propionate accumulated simultaneously.

After 2 hr anoxia, succinate levels fell to about 50% of their maximum value both in the presence and absence of GSA-40 (Fig. 2). The reproducibility of this low level and complex pattern of succinate accumulation and metabolism by *Tubifex* within our set of experimental conditions is in contrast to the results obtained by various authors.

Schöttler and Schroff (1976) reported a continuous increase of succinate concentration up to 150 μmol/g W_d until 48 hr of anoxia in *Tubifex* held at 16°C. In experiments with *Tubifex* involving a gradual decline of oxygen (13°C; Seuß *et al.*, 1983), succinate levels of about 45 μmol/g W_d remained relatively stable between 4 and 48 hr of anoxia. In both cases streptomycin and penicillin were added to the incubation medium. Aerobic metabolism of the oligochaete *L. variegatus* is decreased by 30% after addition of these antibiotics (Gnaiger, 1983a) and any experiment has to be viewed critically where the effect of the added drugs has not been tested for. Putzer (1984) observed a rapid accumulation of succinate restricted to the first 2 hr of anoxia in *L. variegatus*, in agreement with the present results on *Tubifex* studied under the same experimental conditions.

The lactate pathway was not stimulated by the addition of GSA-40 solution (Table 2). The lactic acid pathway, however, is activated by electrical stimulation inducing locomotory activity in *L. variegatus* (Putzer, 1984), in the same way as described for the leech *Hirudo medicinalis* (Zebe *et al.*, 1981). This indicates that the increased glycolytic flux observed as a result of GSA-40 is not due to the induction of high locomotory activity. In thermodynamic terms, the 60% efficient ATP production characterizes the lactic pathway as a *high power* mechanism, whereas the 80–90% efficient ATP production associated with propionate illustrates the extremely *high economy* of this long-endurance anaerobic pathway (Gnaiger, 1983b). In this context it is interesting to note that despite the thermodynamic exclusion principle according to which high efficiency and high rate are mutually exclusive (Gnaiger, 1983b), the stimulating agent GSA-40 is well able to increase the rate of the extremely efficient propionate pathway without simultaneously setting free the high power potential of the costly lactic acid glycolysis.

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