Metabolism and energetics in squid (*Illex illecebrosus*, *Loligo pealei*) during muscular fatigue and recovery

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Pörtner, H. O., D. M. Webber, R. K. O'Dor, and R. G. Boutilier. Metabolism and energetics in squid (*Illex illecebrosus*, *Loligo pealei*) during muscular fatigue and recovery. *Am. J. Physiol.* 265 (*Regulatory Integrative Comp. Physiol.* 34): R157–R165, 1993.—The concentrations of intermediate and end products of anaerobic energy metabolism and of free amino acids were determined in mantle musculature and blood sampled from cannulated, unrestrained squid (*Loligo pealei*, *Illex illecebrosus*) under control conditions, after fatigue from increasing levels of exercise, and during postexercise recovery. Phosphagen depletion, accumulation of octopine (more so in *Illex* than in *Loligo*), and accumulation of succinate indicate that anaerobic metabolism contributes to energy production before fatigue. Proline was a substrate of metabolism in *Loligo*, as indicated by its depletion in the mantle. In both species, there was no evidence of catabolism of ATP beyond AMP. A comparison of the changes in the free and total levels of adenylates and the phosphagen indicates an earlier detrimental effect of fatigue on the energy status in *Loligo*. The acidosis provoked by octopine formation in *Illex* was demonstrated to promote the use of the phosphagen and to protect the free energy change of ATP such that the anaerobic scope of metabolism during swimming is extended and expressed more in *Illex* than in *Loligo*. In both species, there was no decrease in the sum of phospho-L-arginine, octopine, and L-arginine, and thus no release of octopine from the mantle, thereby supporting our earlier claim that octopine and associated protons are recycled in the mantle tissue. Overall, the metabolic strategy of *Loligo* is much less disturbing for the acid-base status. This strategy and the alternative strategy of *Illex* to keep acidifying protons in the tissue may be important for the protection of hemocyanin function in the two species.

aerobic and anaerobic energy production; alanine; ammonium; cephalopod muscle; free adenosine 5′-diphosphate; free adenosine 5′-monophosphate; free energy change of adenosine 5′-triphosphate hydrolysis; α-glycerophosphate; inorganic phosphate; intracellular pH; octopine; proline; phospho-L-arginine; succinate

THE AEROBIC METABOLISM of squid is the most highly tuned in the world of marine invertebrates. Indeed, their exceptionally large aerobic metabolic rates at rest may serve to explain their comparatively small factorial aerobic scope of exercise (32, 33). Nonetheless, high power outputs during jetting may cause metabolic imbalance, as has been found in fatigued squid, linked to the degradation of the phosphagen and anaerobic glycolysis (13, 45). These findings are not uniform among cephalopods and, even among squid, very likely depend on the mode of life. Whereas marked octopine accumulation has been found in *Loligo vulgaris* (10) and, recently, in *Illex illecebrosus*, minor octopine formation or even none was found in *Loligo pealei* (43, 45). Thus it appears there can be considerable interspecific variation in the anaerobic metabolic scope for activity in such animals.

The present study compares two species of squid (*Illex illecebrosus* and *Loligo pealei*) that have distinctly different lifestyles and modes of swimming: *Loligo* relies more on slow aerobic cruising and undulatory fin movements than does *Illex* (cf. Ref. 43). Previously, we reported octopine concentrations and intracellular acidosis to be much greater in *Illex* than in *Loligo* (43). Our current aim is to elucidate the metabolic basis for such differences by comparing the pathways of energy metabolism available to these animals.

To this end, amino acid metabolism, glycolysis, adenylate catabolism, and phosphagen hydrolysis were examined. We have used our previous measurements of intracellular pH to calculate free ADP and AMP levels, which, together with inorganic phosphate measurements, enables calculation of the Gibbs free energy change for ATP hydrolysis. The results corroborate some of the previous conclusions. They demonstrate differences in the use of glycogen and amino acids between the two species. Some of these results lead us to modify some of the generalized concepts on exercise-induced metabolic events in cephalopods.

MATERIALS AND METHODS

Animals. Squid (*Illex illecebrosus*, 300–500 g; *Loligo pealei*, 200–400 g) were caught by commercial fishermen in St. Margaret's Bay or close to Herring Cove, Nova Scotia, from October to December 1986. The animals were transported to Halifax in plastic bags filled with oxygenated sea water at 2–6°C. There they were held in running sea water at ambient temperatures of 8–15°C. At ambient temperatures of 12–15°C, they were used as soon as they recovered from transport and handling (after 2–4 h). At ambient temperatures <12°C, the animals were brought close to the experimental temperature (15°C) for 12–24 h before being used.

Experimental procedure. All experiments were performed on cannulated, unrestrained squid (cf. Ref. 43). After recovery from surgery, animals were placed in a Beamish-type tunnel respirometer that contained 92 liters of normoxic sea water at 15 ± 0.5°C. Water was continuously circulated through the animal chamber at ~0.07 m/s, with partial replacement by fresh sea water on each circuit to maintain high oxygen tensions. Experiments demonstrated that tissue phosphagen, octopine, and pH levels stabilized after ~1.5 h of recovery from handling, at which time experimentation began. A control blood sample (0.6 ml) was withdrawn via the indwelling catheter using 1-ml syringes. Squid were then exercised tail-first by subjecting them to increasing current speeds; the velocity was increased in steps of 0.07 m/s every 5–10 min until the animals showed the first signs of fatigue (unstable swimming, touching the downstream grid). Thereafter, they were maintained at this speed, which was assumed to be close to the critical swimming velocity, until they collapsed from exhaustion. At the end of the exercise period, the water circulation was reduced to 0.07 m/s, and a second blood sample was withdrawn from the exhausted animals. Some of the exhausted squid were then immediately removed from the respirometer for tissue sampling (see below), whereas others were allowed to recover from exercise, with

ng sampled during the recovery period.
sue sampling from control and recovering animals, the eter was closed and the animal anesthetized by adding f pure ethanol to the water circulation downstream of al so that it was fully mixed as it returned. Animals d to full anesthesia (indicated by the cessation of ven- during 3–5 min with no agitation or startle response. er, the animal was removed from the respirometer and ecapitated. A piece of muscle (6–10 cm long) was im- y excised from the left or right ventral mantle using two ades that had been arranged in parallel at a distance of The excision was made against an aluminium ruler 2.5 nserted into the mantle cavity. The muscle sample was mped immediately (49), wrapped in aluminium foil, d under liquid nitrogen until analyzed.

es and calculations. Tissue samples were ground under rogen using mortar and pestle. Samples of the tissue ere extracted in ice-cold perchloric acid as described nd Newsholme (3). Blood samples were deproteinated ddition of cold perchloric acid (3 M) to a final concen- f 0.6 M. After centrifugation, all perchloric acid ex- re neutralized with cold KOH (5 M) and solid K_2CO_3- (1:6 wt/wt) (38). The precipitate was removed by ation.

ncentrations of most metabolites were analyzed using techniques (4, 27). Octopine, phospho-L-arginine and e were assayed in the extracts according to Grie- al. (14); inorganic phosphate was assayed according r (38). Proline and glycine were estimated by means ated amino acid analysis (Liquimat III, Kontron Eching, Germany) at the Zoology Institute, Depart- Animal Physiology, Heinrich-Heine-Universität, rf, Germany.

f the ADP and AMP present in muscle cells is believed nd to cellular protein (28). Therefore, the levels of free AMP were calculated based on the equilibrium of xinase and myokinase using equilibrium constants for xinase (9) and adenylate kinase (26). The values were for the experimental temperature. Because intracel- vas analyzed in each individual muscle sample (40, 43), lependence of both equilibrium constants, related to proton and magnesium binding of the adenylates and n turnover of the arginine kinase reaction, could also nto account in the calculation procedure. Constants of H^+ binding were used as compiled in Refs. 34 and 41. these data, the Gibbs free energy change was calcu- Refs. 1, 15), assuming constant free magnesium levels (1 mM; cf. Refs. 6, 16) and a free inorganic phosphate background of 2 μmol/g fresh weight (as found in the resting muscle of *Sipunculus nudus*; Pörtner, unpublished observation). For information, *Sipunculus* is a marine invertebrate that possesses muscular levels of phospho-L-arginine as high as squid and forms octopine during exercise. A discussion of the influence of pH on the free energy levels can be found in Ref. 39.

Differences between animals were tested for significance at the 5% level by using Student's *t* test for unpaired samples. Data obtained from animals showing extreme values that differ significantly from the norm were identified by Nalimov's test. This approach justified the separate discussion of results from one squid (*Illex illecebrosus*) with an outstanding swimming performance (cf. Ref. 43).

RESULTS

Critique of methods. The validity of the metabolite data for both control and experimental animals depends on the assumption that the metabolic status is, at most, minimally affected by the anesthesia, sampling, and extraction procedures. The high ratio of the concentration of phospho-L-arginine to the concentrations of phospho-L-arginine plus L-arginine in resting squid (~0.7) indicates that the tissue sampling procedure was effective in preserving the energy status of the tissue. This ratio is higher than that calculated from data reported in previous studies on squid (e.g., Ref. 45) and similar to maximum values seen in *Chlamys opercularis* [0.71, calculated from data by Grieshaber (12)] and in *Sipunculus nudus* [0.81 (35)] during rest. Similar ratios for phosphocreatine over phosphocreatine plus creatine have been found in resting toad skeletal muscle (0.71), supported as being valid by inorganic phosphate levels close to those evaluated from ^{31}P-nuclear magnetic resonance studies (42). In contrast, elevated phosphate levels were found in both *Illex* and *Loligo* (Fig. 3; see Table 2) even under control conditions, which could be viewed to represent an artifactual destruction of tissue high energy phosphates caused by the sampling and extraction procedure. However, the above comparison and the validity of the phosphagen data rather suggest that inorganic phosphate stores exist that were mobilized during the extraction procedure and led to an overestimate of these levels under control conditions.

Illex illecebrosus. During intense muscular activity

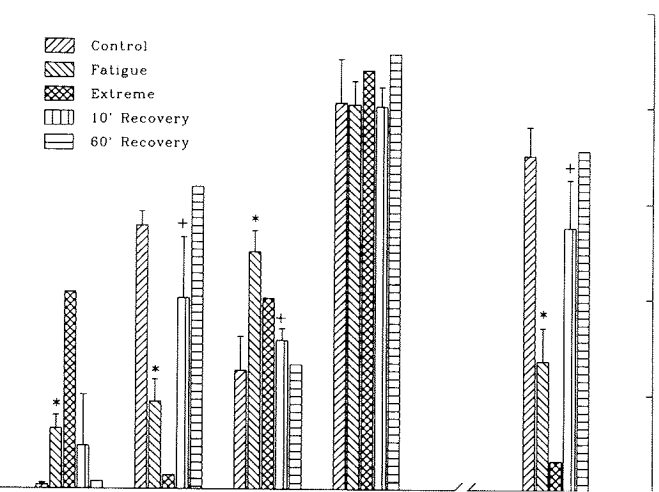

Fig. 1. Octopine, phospho-L-arginine (PLA), and L-arginine (L-Arg) levels and the sum of the concentrations of L-Arg-containing metabolites (ΣArg) under control conditions, after fatiguing exercise, and during subsequent recovery in mantle musculature of the squid, *Illex illecebrosus*. Ratio of PLA content over L-Arg plus PLA concentrations reflects use of phosphagen pool during exercise. Extreme values represent metabolite levels found in one animal showing outstanding swimming performance (octopine levels adopted from Ref. 43; for the respective data in *Loligo pealei*, see Table 2). * Significant change during fatigue from exercise. + Significant change during recovery from exercise.

o fatigue, *Illex* accumulated octopine (43) and phospho-L-arginine in its mantle musculature This general trend was extrapolated to an exone animal with an outstanding swimming per-, which is hereafter referred to as the "elite anee Ref. 43). The contribution of anaerobic was also reflected by a small but significant n glucose-6-phosphate levels that was related to t of octopine accumulation. The significant dehospho-L-arginine levels led to a rise in arginine l a drop in the ratio of phospho-L-arginine over of phospho-L-arginine and L-arginine contents. levels in the blood were low and fluctuated intly (43). Exercise-induced changes in all muscle es began to return to control values after 10 min ry and were completely reversed in one specimen after 60 min of recovery. Octopine fluctuations alleled by minor but not significant changes in nts of pyruvate and L-alanine (Table 1; D-alanot detectable in both *Illex* or *Loligo*). Changes nine levels were smaller than those expected ospho-L-arginine degradation. However, the content of metabolites containing an L-arginine calculated from octopine, phospho-L-arginine, inine concentrations) did not change during the nd recovery periods.

ncentrations of metabolites, which serve to inatabolism of amino acids and anaerobic mito- metabolism, also changed during fatiguing exd recovery in *Illex* (Fig. 2, Table 1), with values again being found in the elite animal. phosphate and succinate concentrations insignificantly, whereas malate levels remained d. Although the levels of glutamate, glutamine, rtate appeared to decrease during the exercise he changes were not significant. Proline and vels were highly variable, although, as with the α-ketoglutarate and ammonium in blood and ey appeared unchanged during the exercise and periods.

epletion led to significantly elevated AMP levels. the adenylates were severely affected by fatigise only in the high-performance animal, with ATP levels and elevated ADP and AMP concen-Fig. 3). This animal also incorporated a severe intracellular acidosis, with pH decreasing from 7.4 to ~6.8 (43). Further metabolization of the adenylates (e.g., from AMP to IMP) was not found, as indicated by the constant sum of adenylate concentrations under all conditions (Fig. 3). Inorganic phosphate concentrations were high under control conditions (see above) but increased stoichiometrically, largely following the changes in phosphagen levels in all exercising and recovering specimens.

The calculation of free ADP revealed concentrations that were significantly (80%) below the total concentrations measured in control animals. In addition, the free ADP increased significantly during fatigue in all animals. The discrepancy between the total measured concentrations and those calculated as free ADP was reduced during exercise (up to 70% free) but became larger once again during recovery (Fig. 4 and Table 2). Calculated and measured AMP values were even closer, but only after exercise, whereas only a small fraction (~20%) was found to be free AMP under control conditions and during recovery.

The levels of free ATP, ADP, and inorganic phosphate represent the Gibbs free energy change ($dG/d\xi$) for ATP hydrolysis valid under the respective conditions (considering intracellular pH values). Starting from 55 kJ/mol, $dG/d\xi$ decreased by >10 kJ/mol during fatigue; the minimum value (41 kJ/mol) was found in the elite animal (Fig. 4). A rapid increase occurred during recovery.

Loligo pealei. A more limited data set, available for *Loligo pealei*, is compiled in Table 2 to illustrate prevailing differences between the two species. The general trends were the same as for *Illex*, with some exceptions. Resting levels of pyruvate and glucose-6-phosphate were lower in *Loligo* than in *Illex* (Tables 1 and 2), and this may account for the much lower octopine formation seen during exhaustive exercise in the former (Table 2, Fig. 1). While *Loligo* had somewhat lower concentrations of phospho-L-arginine and somewhat higher levels of ATP at rest, the extent of phospho-L-arginine depletion was as great as in the high-performance specimen of *Illex* (as indicated by the maximum drop in the ratio of phospho-L-arginine over the sum of phospho-L-arginine plus L-arginine concentrations). However, the depletion of ATP during exercise was more severe in *Loligo* and correlated with a larger accumulation of AMP. Fully 82% of the ADP and 100% of the AMP were found to be free with

Selected metabolites in blood and mantle tissue of the squid, Illex illecebrosus, under control conditions, uing exercise, and after 10 and 60 min of subsequent recovery

	Pyruvate	α-KG	L-Alanine	Proline	Glycine	Glutamine	NH_4^+		G-6-P
							Mantle	Blood	
(5)	0.11	0.05	2.80	74.0	4.77	0.77	0.58	0.29	0.15
	±0.09	±0.02	±2.46	±36.9	±3.73	±0.86	±0.26	±0.21	±0.03
(3)	0.15	0.06	3.32	86.8	4.77	0.21	0.61	0.24	0.22*
	±0.05	±0.02	±0.05	±21.2	±1.52	±0.16	±0.03	±0.03	±0.01
	0.29	0.05	7.40	95.2	6.49	0.30	0.73	0.39	0.49
y									
n (5)	0.15	0.06	2.23	77.6	6.78	0.55	0.53	0.15	0.21
	±0.08	±0.01	±1.21	±39.0	±3.40	±0.44	±0.14	±0.09	±0.11
n	0.09	0.06	3.09	75.9	2.13		0.48	0.03	0.35

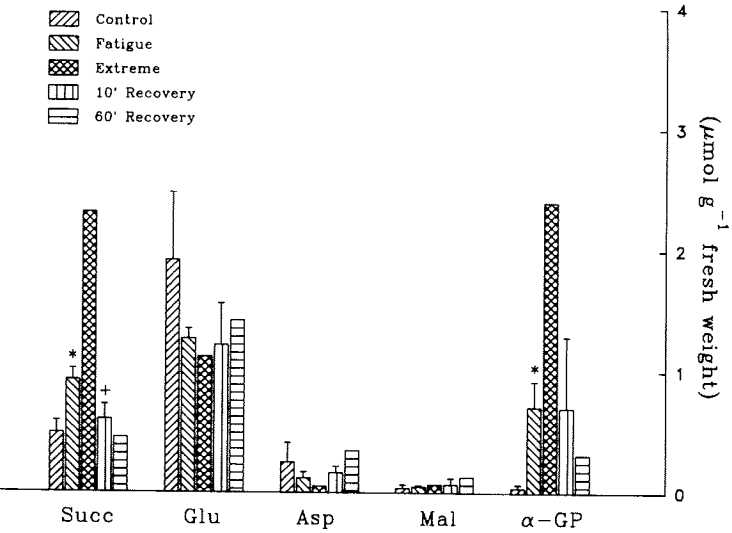

Fig. 2. Concentrations of succinate (Succ), glutamate (Glu), aspartate (Asp), malate (Mal), and α-glycerophosphate (α-GP) under control conditions, after fatiguing exercise, and during subsequent recovery in mantle musculature of the squid, *Illex illecebrosus* (cf. Fig. 1; for respective data in *Loligo pealei*, see Table 2).

)P and AMP levels during fatigue and recovery). Free energy values were the same in *Loligo* as in der control conditions. Fatigued *Loligo* reached minimum free energy values to those observed in -performance *Illex* specimen.

ıtrast to *Illex*, α-glycerophosphate remained low he exercise and recovery periods in *Loligo*, but e reached much higher levels (Table 2, Fig. 2). proline levels were similar in both species, but as to *Illex*, proline decreased during exercise and re so during the recovery period in *Loligo*. L-Ala- d glycine were not only present at much higher rations in *Loligo*, but unlike *Illex*, they accumuring the exercise period. Alanine levels in the mained <0.2 mM in both species. Mantle ammo- slightly during exercise in *Loligo*, whereas am- the blood remained more or less constant, as it *lex*.

ION

olic fuels. The correlation between nitrogen exoxygen consumption, and swimming speed in 22) and the resulting oxygen-to-nitrogen ratios suggest that steady-state aerobic metabolism in adult squid is mainly fueled by protein and amino acids. Amino acids are readily metabolized by squid tissues like the heart (cf. Ref. 21), although some tissues also depend on provision of blood glucose (e.g., the heart; Ref. 7). During intense muscular exercise, the energy metabolism of squid mantle is also fueled by glycogen stores whose utilization gives rise to octopine formation (11, 18, 47). In the present study, octopine and alanine accumulation and the use of amino acids such as aspartate, glutamate, glutamine, and proline indicate a coordinated use of glycogen and amino acids under these conditions. Substrate preferences may differ, however, and the capacity for octopine formation is likely to be lower in *Loligo* than in *Illex*, as evidenced by the lower levels of glucose-6-phosphate, pyruvate, and octopine in the mantle tissue of the former. As outlined by Pörtner et al. (43), this may be related to differences in swimming behavior between the two species and reflects the lower oxygen debt seen in loligids (33).

Aerobic vs. anaerobic metabolism. Anaerobic metabolism during muscular exercise (functional anaerobiosis) in squid could just indicate anaerobic on top of maximum

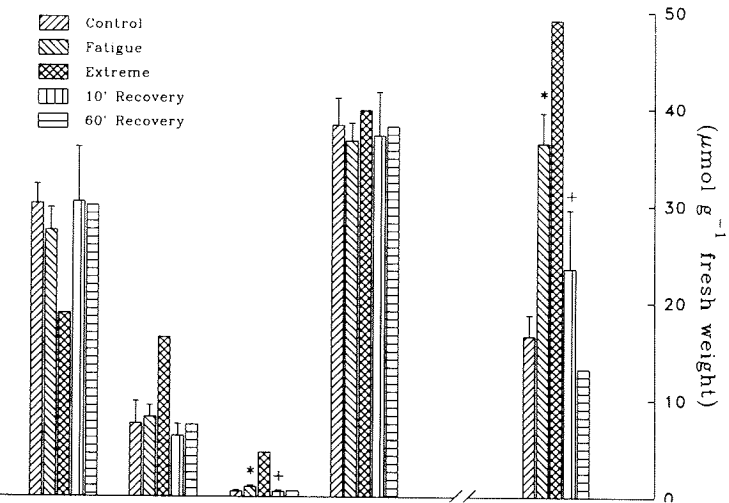

Fig. 3. Measured concentrations of ATP, ADP, and AMP and the sum of the levels of the three adenylates (ΣAde) under control conditions, after fatiguing exercise, and during subsequent recovery in mantle musculature of the squid, *Illex illecebrosus*. Changes in content of P_i mirror depletion of high-energy phosphates during exercise period (cf. Fig. 1; for respective data in *Loligo pealei*, see Table 2).

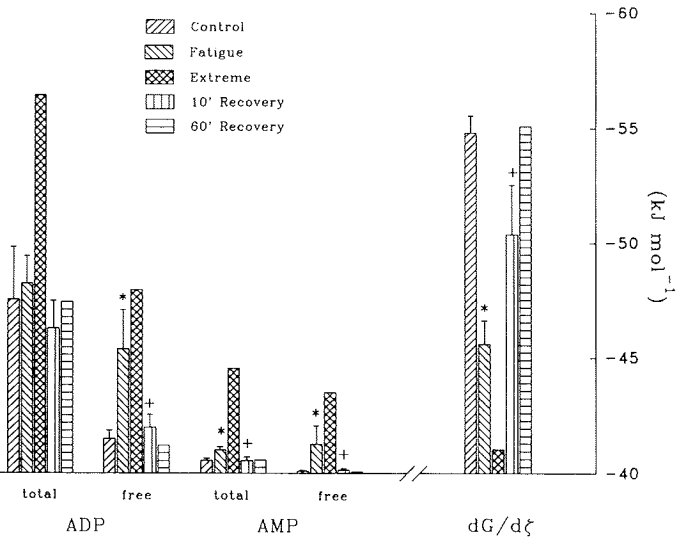

Fig. 4. Comparison of calculated levels of free ADP and free AMP with (total) concentrations measured under control conditions, after fatiguing exercise, and during subsequent recovery in mantle musculature of the squid, *Illex illecebrosus* (cf. Fig. 1; for respective data in *Loligo pealei*, see Table 2). Free ADP, P_i, and ATP values yield free energy change ($dG/d\zeta$) of ATP under in vivo conditions.

ergy production. In squid, this pattern is likely e only during short-term bursts of maximum 5). The metabolic changes seen in fatigued *Illex* are similar to those observed in squid exposed ia, with accumulation of α-glycerophosphate *Illex*) and succinate (20). Accumulation of α- osphate could signal a failure of its shuttle of ivalents into mitochondria caused by a block of atory chain. Despite the rather small changes in glycerophosphate accumulation may reflect an ly reduced redox status of the cytosol. If this e prominent in *Illex* than in *Loligo* it could be e explanation for the larger octopine formation the former. Based on a mass action control of reductases (24), increases in NADH, arginine, ate concentrations constitute the driving forces ne formation (10). Thus lower pyruvate concen- nd possibly also lower NADH levels in *Loligo*, lude octopine formation and otherwise enable ction of alanine (see below).

te is likely to have accumulated because of formation by fumarate reductase activity. The eleton of succinate may originate from amino proline, etc. (see below), which might partly e higher succinate levels reached in *Loligo*. Suc- umulation has also been observed during exer- *ligo vulgaris* (13). Because of the low oxygen- capacity of the hemocyanin, insufficient blood ansport to the working muscle is most likely to ochondria to become anaerobic (32, 37, 43).

acid catabolism. Proline levels in our animals iderably higher than those reported previously (45) and *Illex* (31). This may reflect differences mpling procedures, where anesthesia before ue sampling from fully recovered animals leaves ile metabolites like phospho-L-arginine unaf- e above). However, rapid sampling precludes ction between the aerobic outer layers and the erobic middle layers of the mantle tissue (29); ities of proline oxidation are lower in the mid- an in the outer mantle. Because the differences trends should nonetheless become apparent based on the measurement of the mean changes in metabolite levels.

Evidence from our previous study, and from the data herein, suggests that the anaerobic scope of *Loligo* is far less than that of *Illex*. If so, it seems reasonable to suppose that *Loligo* might therefore place greater emphasis on aerobic energy production to offset muscular fatigue. Indeed, during exhausting exercise, *Loligo* uses proline stores (Table 2; Ref. 45), whereas *Illex* may not (Table 1). Although Mommsen et al. (31) found amino acid metabolism to be important in *Illex*, the only evidence for this in the present study was the gradual use of aspartate and glutamate (glutamine) in mantle tissue (Fig. 3, Table 1). The extent to which proline is consumed by the mantle may differ between squid species (30); for example, proline is used by the squid *Allotheutis* (19) in much the same fashion as in exercising *Loligo*. However, unlike *Allotheutis*, proline depletion in *Loligo* was correlated with the accumulation of alanine. Although a comparatively small amount of alanine accumulation occurred in the elite performing *Illex*, this was not correlated with utilization of proline.

One possible explanation for alanine formation could be that glycolytic pyruvate is being used as a final acceptor of the amino group (2). The transfer of amino groups to pyruvate could suggest that, under non-steady-state exercise conditions, glutamate oxidation is insufficient. In the context of elevated succinate levels, which indicate insufficient oxygen supply to the tissues, transamination may be preferred when glutamate dehydrogenase is inhibited by elevated NADH-to-NAD ratios (44).

Glycine accumulation also occurred in *Loligo*, was insignificant in *Illex* (present study), and was not observed in *Alloteuthis* (19). A trend for glycine to accumulate is also apparent in the data of Storey and Storey (45). The metabolic background of glycine accumulation remains unexplained. It could reflect an imbalanced use or uptake of amino acids after protein hydrolysis during the non-steady-state exercise situation. Actually, an imbalanced use of amino acids after protein breakdown might explain that both alanine and glycine accumulate when proline is

	G-6-P	Pyruvate	Octopine	PLA	L-Arginine	ΣArginine	Succinate	Malate	Glycine	ATP	ADP	AMP	ΣAdenylate	[PLA]/[PLA] + [L-ARG]	P_i	α-GP
Control	0.04	0.06	0.54	26.5	15.3	42.4	0.41	<0.04	7.58	0.96	0.15	8.70		0.63	16.0	0.39
Fatigue (3)	0.04	0.10	2.98	4.1	43.3	50.8	3.69	<0.04	3.73	3.35	2.22	9.31		0.09	51.9	0.35
	±0.01	±0.01	±0.86	±0.4	±1.9	±2.1	±0.11		±0.20	±0.03	±0.17	±0.06		±0.01	±0.4	±0.11
Recovery,	0.05	0.06	1.88	16.4*	29.6*	47.9	0.85*	<0.04	6.79*	1.62*	0.28*	8.68		0.35*	32.1*	0.10*
10 min (3)	±0.01	±0.02	±1.41	±6.2	±2.2	±9.4	±0.46		±1.07	±0.54	±0.14	±0.42		±0.08	±0.7	±0.08

	Glutamate	Glutamine	Aspartate	Proline	L-Alanine	Glycine	NH_i^+ Mantle	NH_i^+ Blood	Free ADP	%Free	Free AMP	%Free	dG/dξ
Control	1.67	0.11	0.38	71.3	14.7	35.2	0.44	0.30 ±0.15(7)	0.33	34.3	0.02	10.5	−54.5
Fatigue (3)	1.50 ±0.66	0.20 ±0.08	0.70 ±0.45	44.0 ±9.1	35.4 ±17.4	84.2 ±21.2	0.92 ±0.18	0.31 ±0.15	2.76 ±0.03	82.3 ±0.2	2.28 ±0.08	102.8 ±4.5	−40.5 ±0.1
Recovery, 10 min (3)	1.32 ±0.16	0.13 ±0.03	0.39 ±0.20	19.9* ±9.7	27.1 ±13.1	65.4 ±6.9	0.98 ±0.05	0.19 ±0.07	1.22* ±0.13	79.0 ±16.4	0.26* ±0.10	97.1 ±9.9	−46.2* ±0.7

Values are means ± SE; (*n*), no. of animals. Concentrations are given in micromoles per gram fresh weight or micromoles per milliliter blood, respectively. G-6-P, glucose-6-phosphate; PLA, phospho-L-arginine; ΣArginine, sum of L-arginine-containing metabolites; Σadenylate, sum of adenylate concentrations; α-GP, α-glycerophosphate; dG/dξ, ATP free energy values (kJ/mol). *Significant change during recovery from fatigue.

nine formation contributes to anaerobic ATP formation (see Table 3).

Proline levels continued to decrease during recovery in *Loligo*, although the decline was no longer correlated with the net accumulation of alanine. Proline may, therefore, be supporting the replenishment of glycogen stores under these conditions (17). The reason for the continued breakdown of proline stores during recovery might be related to exercise-induced accumulation of free ADP and AMP, which are known activators of a highly potent glutamate dehydrogenase in this species (44).

If the changes in octopine and L-alanine levels are compared with the observed changes in the acid-base status (43), it becomes apparent that only octopine formation causes an acidosis. Net proline catabolization is proton consuming (36), and the release of ammonia to the environment (or the simultaneous formation of alanine by transamination) would compensate for this proton uptake (34). In fact, ammonia levels remain more or less constant during exercise.

Phosphagen breakdown and adenylate metabolism. In accordance with a gradual failure of aerobic ATP production with increasing performance, a depletion of ATP occurred during exercise in both squid species. Although ATP degradation led to an accumulation of AMP, especially in *Loligo*, there is no evidence for a degradation beyond AMP, since, despite long-term exercise, the summed concentrations of all adenylates remained constant. A delayed catabolization of AMP compared with vertebrate muscle had also been observed during an analysis of long-term postmortem metabolism in *Illex illecebrosus* by Langille and Gill (25).

The total measured amounts of ADP or AMP do not reflect the free concentrations of the two metabolites. Because intracellular pH values are available for these animals (43), free ADP and AMP levels can be calculated. Comparison of measured and calculated data (Fig. 4) suggests that the bulk of these substances is bound under control conditions, but this is no longer the case if ADP and AMP accumulate, as they do during exercise, when most of the ADP (up to 80%) and almost all of the AMP remain free. Apparently, the binding capacity for ADP is larger than for AMP. Since most of the accumulated ADP and almost all of the AMP is found in the free form during exercise, it will become effective at the enzyme level, e.g., to stimulate glycolysis under these conditions as suggested by Storey and Storey (45; cf. Ref. 18). AMP overrides the inhibition of glycolysis by NADH, which is a special feature in cephalopods (47), as soon as ATP depletion occurs (see below), thus responding to energy requirements in excess of aerobic energy production. This type of regulation might permit a tighter coupling of energy consumption and aerobic energy production in these highly tuned animals.

Higher free ADP levels were reached in *Loligo* than in *Illex*. This difference reflects the importance that free ADP has in triggering transphosphorylation of phospho-L-arginine (PLA), especially in *Loligo*. According to the equation $K_{eq} = [\text{L-Arg}][\text{MgATP}]/[\text{PLA}][\text{MgADP}][\text{H}^+]$

(increased [H^+]) and/or by the catabolization of L-arginine and/or by an accumulation of free MgADP. Since no net depletion of L-arginine metabolites was found in either species of squid, only octopine formation decreases the level of this amino acid and, thereby, supports phospho-L-arginine transphosphorylation and the concomitant buffering of ATP values (11). It could previously be demonstrated that octopine formation causes an intracellular acidosis in squid (43) that, in the case of *Illex*, will support PLA depletion. In *Loligo*, ADP accumulation is the only means to exploit the phosphagen, meaning that ATP may be less well buffered in this species. Actually, less ATP is degraded to ADP and AMP in *Illex* than in *Loligo*. As a corollary, the ATP level is less protected without than with a concomitant acidosis under conditions when phosphagen is available.

The data in Table 3 allow further quantification of these considerations by comparing the extent of anaerobic ATP formation with the intracellular pH and free ADP values seen in both species. The free energy levels of ATP hydrolysis represent the driving force for any ATPase reaction and quantify the energy status of the tissues. Starting from higher levels of PLA, free energy values of ATP remain higher in *Illex* than in *Loligo*, with a similar use of the phosphagen and a similar extent of anaerobic ATP formation (excluding alanine) in the two species. Minimal free energy values are similar in both species, but *Illex* can produce more ATP anaerobically and use more phosphagen by *1*) forming octopine and protons and *2*) accumulating less free ADP. The similarity of minimal free energy values is due to the fact that the free energy of ATP hydrolysis (with unchanged concentrations of ATP, ADP, and P_i) falls with pH until it reaches a broad minimum at pH values of ~6.6–6.7 (for a depiction, see Ref. 39). Further studies should investigate whether these minimal free energy values contribute to fatigue in these species. They are actually far below those required for the maintenance of control steady-state function of essential ATPases in muscle tissue [myosin ATPase, Na^+-K^+-ATPase, and especially the sarcoplasmic Ca^{2+}-ATPase (23; cf. Ref. 39)] and thus will be accompanied by a change in the respective distribution equilibria of ions.

In vertebrate muscle, an acidosis is suggested to stimulate AMP deaminase, thus minimizing the accumulation of AMP (8). This reaction is obviously less important in squid muscle, since AMP accumulates under these conditions, meaning also that ADP accumulation is not prevented or reduced by the adenylate kinase reaction. As a corollary, although gradual differences have become apparent between the two squid species, ADP may act as the most important parameter in triggering phospho-L-arginine depletion, and this may be the case in a number of molluscs. In pectenids, for example, octopine formation is either absent or only minor during activity but eventually occurs during recovery (12), ADP being the only factor that could elicit transphosphorylation from the phosphagen.

L-arginine metabolism: fate of octopine. The decrease in the sum of L-arginine metabolites in fatigued *Sepia officinalis* led to the view that some of the tissue arginine may be lost into the blood as octopine and then oxidized by other tissues like the brain or the ventricle analogous to the vertebrate Cori cycle. This conclusion was also based on minor amounts of octopine found in the blood (46–48). However, even if a release of up to 17% of the arginine stores occurred (47), this amount is still small in comparison to the amounts retained within the mantle tissue. The changes in blood octopine levels were also very small (47) and may simply represent a minor leak. More recent evidence demonstrates that urea formation may occur from arginine and that the catabolism of ornithine via pyrroline-5-carboxylate may explain the decrease of L-arginine metabolites observed in *Sepia* (19, 31). *Illex* swum to collapse at supracritical speeds showed no depletion of the sum of arginine metabolites in the tissue. The rates of urea excretion found by Hoeger et al. (22) were not correlated with the exercise level of the animals but most likely depended on their nutritional status. As a corollary, the free mantle arginine store does not readily fuel exercise metabolism in squid.

Illex also showed only minimal changes in blood octopine. The available data suggest the same to be true for *Loligo*. Both species retained protons and octopine in the tissue (43), emphasizing that octopine is largely used as a "depository" for pyruvate and arginine, which are subsequently recycled within muscle cells. Rapid uptake of octopine from the blood might be possible via an amino acid carrier responsible for the cellular uptake of nutritional arginine (19, 48), thus mimicking the existence of an octopine cycle analogous to the Cori cycle in the vertebrates. Brain type ODH isozymes, which have been used to argue for the existence of a Cori cycle, may simply reflect the aerobic nature of this organ, with the potential to minimize octopine formation and to readily oxidize octopine accidentally taken up.

Table 3. *Comparison of degradation of phospho-L-arginine, accumulation of L-arginine, anaerobic ATP formation by catabolism, pH_i, free ADP levels, ATP free energy change values, and ratio of [PLA] to [L-Arg] in fatigued squid*

	ΔPLA	ΔL-Arg	$\dot{M}_{ATP}$	pH_i	Free ADP	dG/dζ	[PLA]/[L-Arg]
Illex	−22.2	+11.3	33.2 [33.2]	7.17	1.1	−45.1	0.37
	(−31.5)	(+14.9)	(73.0[78.9])	(6.78)	(1.6)	(−41.0)	(0.07)
Loligo	−22.5	+28.0	35.4 [66.5]	7.22	2.8	−40.5	0.09

$\dot{M}_{ATP}$, anaerobic ATP formation by catabolism [including alanine formation (in brackets)]; dG/dζ, ATP free energy change values (kJ/mol). pH_i values, see Ref. 43. Data on *Illex* include the high-performance animal (in parentheses), which reached similarly low free energy values as fatigued *Loligo*.

mical nature of the octopine molecule also ex-
t it is handled similar to lactate. Lactate move-
ween intra- and extracellular space are not only
concentration gradients but even more so by
vs. extracellular pH gradient, which is high
tebrates but not in molluscs (39, 43). Generally,
driving force is not effective for the release of
m the tissue, and a concentration-dependent
by diffusion is most unlikely because of the
ic character of the octopine molecule at phys-
H (34).

ions. Based on the comparatively reduced oc-
mation in exercising *Loligo*, Pörtner et al. (43)
that the ability to extend the swimming per-
beyond the aerobic scope may be less expressed
han in *Illex*. This conclusion is corroborated by
t analysis, as evidenced by the earlier detrimen-
f fatigue on the energy status in *Loligo*, earlier
fter less anaerobic ATP production. In both
ere is no evidence for release of octopine from
e important enough to cause a decrease in the
rginine-containing metabolites. Octopine and
protons are almost certainly recycled in the
sue. The depletion of proline stores during ex-
oligo is in accordance with a greater emphasis
metabolism in this species. The background
s of the concomitant accumulation of alanine
e remain to be investigated. Although unfavor-
s overall energy status, the advantage of the
Loligo is that it is much less disturbing for the
status and may possibly be important with re-
e protection of hemocyanin function in these
or the same reason, *Illex* is able to keep the bulk
ic protons within the mantle tissue, thus min-
e risk of an extracellular acidosis and the asso-
l reduction of oxygen binding to the hemocy-
43).

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