epidermoid growth factor (*17*) have both been described in submaxillary glands of male mice. Epidermoid growth factor, the polypeptide that promotes precocious eye opening and toothbed eruption in mice, has also been described in human urine (*18*), and gastrin-like immunoreactivity has been found in bovine parotid glands and in saliva (*19*). Our studies, reported here, dealing with the presence and release of a large glucagon-like material from the submaxillary gland, even possibly a proglucagon, lay the groundwork for additional investigations to determine the biologic significance of these findings.

A. M. LAWRENCE, S. TAN
S. HOJVAT, L. KIRSTEINS

Research and Medical Services, Veterans Administration Hospital, Hines, Illinois 60141

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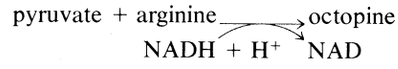
Octopine as an End Product of Anaerobic Glycolysis in the Chambered Nautilus

Abstract. *The terminal step in the anaerobic glycolysis of muscle in the chambered nautilus,* Nautilus pompilius, *is not pyruvate reduction to lactate as in vertebrate muscle. Instead of lactate dehydrogenase, these organisms utilize octopine dehydrogenase (E.C. 1.5.1.11), catalyzing the reductive condensation of pyruvate and arginine, which is dependent on the reduced form of nicotinamide adenine dinucleotide, to form octopine and the oxidized form of the coenzyme. The kinetic properties of octopine dehydrogenase favor the production of octopine, which accumulates under a variety of conditions.*

Across the animal kingdom, there is the problem of producing mechanical work from chemical stores under both aerobic and anaerobic conditions. In the vertebrates, the efficiency of conversion is high during aerobic glycolysis, 38 moles of adenosine triphosphate (ATP) being produced for each mole of glucose 6-phosphate (G6P) oxidized. Under anaerobic conditions, the efficiency drops to 3 moles of ATP per mole of G6P converted to lactate, but the accumulated lactate can be recycled when or where aerobic conditions prevail and so be fully oxidized. Do important invertebrate groups like the cephalopods follow a similar strategy or have they evolved different mechanisms for coping with the differing conditions under which work is demanded by their life-styles?

Until recently not much was known about metabolic organization in the cephalopods, even though the squid is a favorite experimental material for electrophysiologists and the octopus has been used to open new frontiers in comparative animal behavior. This situation was at least partially remedied in the fall of 1973 when a group of us had the opportunity to study a fast-swimming predaceous squid, *Symplectoteuthis* (*1*). We found the mantle muscle packed with mitochondria and displaying an extremely aerobic metabolism primed by a functional α-glycerophosphate cycle, with both cytoplasmic α-glycerophosphate dehydrogenase (E.C. 1.1.1.8) (α-GPDH) and the mitochondrial α-glycerophosphate oxidase occurring in substantial activities (*1*). On the other hand, the anaerobic capabilities of this muscle did not depend on lactate dehydrogenase, which is in fact absent, but upon octopine dehydrogenase (ODH) (E.C. 1.5.1.11), which catalyzes the reductive condensation of pyruvate and arginine (*2*) (NAD is nicotinamide adenine dinucleotide; NADH, reduced form of NAD):



Since simultaneous function of ODH and α-GPDH would lead to a carbon and energy drain off main-line glycolysis (*3*), it was not surprising to find (i) that under most physiological conditions α-GPDH activity could exceed that of ODH by a factor of nearly 100:1 (*4*) and (ii) that therefore octopine typically did not accumulate in fast-swimming cephalopods (*5*). By implication, the reverse of this situation should prevail in more anaerobic cephalopods. Our first opportunity to test these ideas came during the expedition of R.V. *Alpha Helix* to the Philippines, where we studied the chambered nautilus (*Nautilus pompilius*), a phylogenetically ancient animal, shelled and slow swimming. We found α-GPDH at barely detectable levels but ODH at high activities and displaying kinetic properties consistent with function under physiological substrate concentrations. Predictably, octopine accumulation in muscle could be demonstrated under a variety of situations.

Nautilus ODH [see (*6*) for assay method] occurs in highest activities in spadix, funnel, and retractor muscles [about 44, 42, and 38 μmole of product formed per minute per gram wet weight (*p*H 7 and 25°C)]; in the heart, the activities are reduced to about one-fourth of these values. With electron microscopy we observed that mitochondria are least abundant in the spadix and most abundant in the heart, but in none of these muscles are they as abundant as in squid muscle (*1*, *7*). In contrast to the squid, where the ratio of α-GPDH to ODH is high, α-GPDH in the *Nautilus* spadix occurs at less than 1/40 the ODH activity; in retractor muscle it is about 1/13 the ODH activity. This arrangement is metabolically "sensible," since simultaneous function of both enzymes would lead to a carbon drain from glycolysis (to α-glycerophosphate) and to a serious drop in glycolytic energy yield. The activity ratios in *Nautilus* are of course just opposite to those found in the squid (*1*). Thus the absolute activity of ODH as well as the activity ratio of ODH to α-GPDH seem to roughly parallel the anaerobic capabilities of the muscle and indeed of the whole animal,

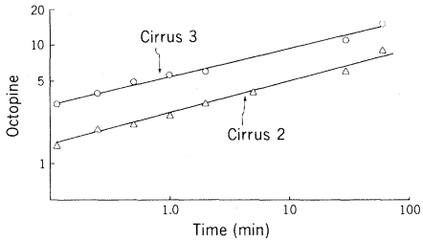


Fig. 1. Two parallel experiments in which cirrus 2 and cirrus 3 from the spadix of two different individuals were used. Each cirrus was given a single stimulus of 15-second duration (*12*) while contracting against a 50-g load, then allowed to recover at room temperature in air. The muscles were kept moist during the recovery period and as O_2 is normally delivered to these by the blood it is assumed that they were essentially anoxic. Octopine concentrations, given in micromoles per gram of tissue wet weight were determined in small samples of muscle (weight, 100 to 300 mg) taken from each cirrus at the times shown. Octopine concentration in control cirri also increased over this time interval, but by only about 30 percent. A log-log plot is used only for purposes of data compression.

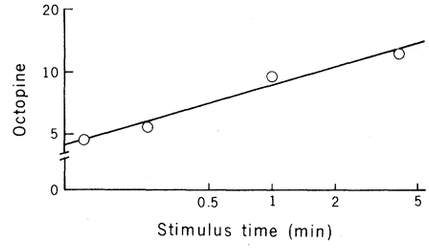


Fig. 2. Octopine production during increasing periods of "burst" work. A single retractor muscle was excised from a cold-anesthetized nautilus and cut into four similar-sized strips. Each strip was then stimulated (*12*) to contract against a constant 50-g work load for periods of 5, 15, 60, and 240 seconds. At 15 seconds after completion of this work "burst," the muscle was quickly frozen (−80°C in ethanol), and octopine concentrations were determined as described (*11*) and are expressed as micromoles per gram of tissue wet weight. Initial concentration of octopine in the retractor muscle was usually somewhat higher than in similar spadix preparations because of greater stimulation of the muscle during extraction and subsequent manipulations. Similarity of slope compared to the spadix muscle preparations in Fig. 1 is presumed to be fortuitous.

for the bottom-dwelling nautilus appears better able to cope with restricted availability of O_2 than fast-swimming squids (*8*).

In this context, it was interesting to characterize *Nautilus* ODH and to look for accumulation of octopine, the presumed and novel end product of anaerobic glycolysis in these organisms. By electrophoretic criterion (*9*), ODH occurs in two anodally moving forms: the faster form moves at about 1 cm per 4 hours at a voltage potential of 250 volts, a current of 30 ma, 4°C, 12 percent starch gel, *p*H 7.2; the slower migrating form is found only in retractor muscle where it constitutes about 50 percent of the enzyme activity. The faster migrating form is found in all three muscles studied; in the spadix and heart it accounts for nearly all of the ODH activity. In this study, the kinetic properties of only this fast-moving form are reported.

Spadix ODH, like its homolog in other mollusks (*10*), shows a neutral *p*H optimum when assayed in the forward direction; the optimum for the back reaction is alkaline (about *p*H 9). Some of the key kinetic constants for the forward reaction (see Table 1) indicate (i) that increasing availability of one substrate increases the enzyme affinity for the cosubstrate, an effect constituting the only known control mechanism on this enzyme in *Nautilus* and elsewhere (*10*). (ii) Under physiological concentrations of either substrate (*11*) the Michaelis constant (K_m) for the cosubstrate is lower than its physiological levels are; that is, the enzyme can compete well for both substrates and one would therefore predict octopine accumulation in vivo at least under some circumstances. This is indeed observed.

Our first attempts at assaying octopine levels in *Nautilus* muscle were done on intact animals. Merely removing the animal from its shell led to octopine buildup in the retractor muscle to concentrations of 20 to 30 μmole per gram wet weight of muscle (average of five such determinations was 26 μmole). If, however, the nautilus was first chilled in ice seawater so that it was in effect cold anesthetized, and therefore did not struggle during removal from its shell, concentrations of octopine in retractor muscle were much lower, varying between 2 and 5 μmole/g wet weight.

Similar results were obtained with isolated muscles. The spadix was an unusually good system since cirri 2 and 3 are in effect mirror images of each other; therefore, one could serve as a control while the other was stimulated against a constant work load for a period of 4 minutes at 25°C (*12*). In a typical experiment, the octopine concentration in the control cirrus was 3.4 μmole/g while in the experimental muscle after 4 minutes of work it was 14.0 μmole. After an hour, the octopine concentrations in the control cirrus had risen to only 4.8 μmole/g.

Figure 1 shows the time course of octopine accumulation in two separate cirrus preparations following a single stimulus of 15-second duration. As a function of time, octopine concentrations rise to values over 10 μmole/g wet weight.

In a different kind of experiment (see Fig. 2) the retractor was removed from a cold-anesthetized nautilus and sliced into four similar-sized strips. Each strip was then stimulated to contract against a constant work load but for different time periods. Octopine concentrations, determined in muscle samples taken at 15 seconds after the work burst, increased approximately in proportion to the work period (Fig. 2). To our knowledge, these are the only data available showing the accumulation of octopine as an end product during anaerobic work bursts in the cephalopods [see (*10*)].

The final question, to be considered briefly, concerns the subsequent fate of this anaerobic end product. There appear to be two possibilities: (i) Since the ODH reaction is reversible, it is probable that some of the octopine is simply reconverted to pyruvate and arginine upon return to aerobic conditions. (ii) Octopine could diffuse into the blood and be removed to other tissues for subsequent metabolism. Further work is required to fully establish the relative importance of these alternatives.

These results, in summary, indicate that octopine dehydrogenase (i) occurs in substantial activities in *Nautilus* muscles, (ii) displays kinetic properties favoring octopine formation at substrate concentrations well within the physiological range, and (iii) mediates octopine accumulation in muscles under a variety of anaerobic conditions. Cephalopods are thus seen to have evolved enzymatic and metabolic machinery which is different

Table 1. Michaelis constants (K_m values) for pyruvate at varying arginine concentrations and for arginine at varying pyruvate concentrations for *Nautilus* spadix muscle octopine dehydrogenase. Assay was performed in 100 m*M* imidazole buffer (*p*H 7.0 at 25°C) and 0.2 m*M* NADH. The K_m values were determined by double reciprocal plots (1/velocity of reaction versus 1/substrate concentration). Error ± 10 percent. Tissue concentrations were determined as described (*11*): pyruvate 0.1 to 0.9 and arginine 15 to 30 μmole/g wet weight, from ten different muscle preparations.

$K_{m(pyr)}$ (m*M*)	Arginine (m*M*)
1.3	1.5
0.7	6.0
0.3	30.0
$K_{m(arg)}$ (m*M*)	Pyruvate (m*M*)
7.7	0.45
5.3	0.9
3.7	3.0

in details from that of the vertebrates. However, the ground plan in the two groups is similar, for octopine and octopine dehydrogenase are formally analagous to lactate and lactate dehydrogenase in the vertebrates, both enzymes serving to sustain an oxidizing potential during anaerobic glycolysis.

P. W. HOCHACHKA*
P. H. HARTLINE†
J. H. A. FIELDS‡

Bindoy Village, Negros Oriental, The Philippine Republic

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6. The procedure for isolating octopine dehydrogenase was as follows. Animals that were freshly captured in baited traps were removed from their shells, and the desired muscle was quickly excised, then homogenized usually in five or ten parts of 100 m*M* imidazole buffer (*p*H 7.0). The homogenate was spun in an RC-2B Sorvall refrigerated centrifuge at 4°C and 12,000*g* for 20 minutes. The supernatant solution contained essentially all extractable octopine dehydrogenase. For studies of its kinetic properties the enzyme was partially purified and prepared as described by Fields *et al.* (*10*). The enzyme was assayed in either a Unicam SP 1800 recording spectrophotometer or a Beckman DB recording instrument by following the change in optical density at 340 nm due to either NADH oxidation (in the forward direction) or NAD^+ reduction (in the back reaction). Assay conditions for the forward reaction: 0.2 m*M* NADH, varying pyruvate or arginine concentrations, 100 m*M* imidazole buffer (*p*H 7.0) at 25°C and a pressure of 1 atm; for the back reaction: 1.0 m*M* NAD^+, varying octopine concentrations, and 100 m*M* tris-HCl buffer at varying *p*H.
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11. A partially purified preparation of *Nautilus* ODH (free of contaminating enzymes such as lactate dehydrogenase, malate dehydrogenase, α-glycerophosphate dehydrogenase, isocitrate dehydrogenase, malic enzyme, or glucose 6-phosphate dehydrogenase) was used to assay octopine concentrations by procedures described by J. R. Williamson and B. E. Corky [*Methods Enzymol.* **13**, 434 (1969)]. Pyruvate levels in perchloric acid extracts of rapidly frozen tissues were assayed with the use of purified lactate dehydrogenase (Sigma). Arginine concentrations were determined on an amino analyzer by standard procedures.
12. Muscle preparations, either dissected cirrus muscle from the spadix or strips of retractor muscle, were hung vertically at room temperature (24°C) attached to a constant 50-g weight as a work load. The muscles were kept in seawater during most manipulations but stimulation always occurred in air. Stimulus conditions: 150 volts, 10-msec direct electrical stimulation of muscle at 10 hertz, for time periods described in the figure lengends. One 150-volt, 10-msec biphasic stimulus pulse was more than twice the intensity that evoked maximal twitch amplitude in a rested muscle strip. A 10-hertz stimulation caused smooth tetanic contraction of the muscle to between 66 and 50 percent of its relaxed length under 50-g loading. A continuously tetanized muscle relaxed to within 5 percent of its original length in about 15 minutes, and thereafter could produce no further work against the 50-g weight even after a 10-minute rest period.
13. The R.V. *Alpha Helix* expedition was funded by the NSF. P.W.H. was supported by an operating grant from the NRC (Canada); J.H.A.F. was an NRC predoctoral scholar; P.H.H. was supported by NIH (EY 01539). Especial thanks are due Dr. Jim Redmond for inviting us on his nautilus expedition and to Wilson Vailoces and his family and friends for introducing us to the haunts of the *Nautilus*. Dr. John Arnold introduced us to the spadix.

* Permanent address: Department of Zoology, University of British Columbia, Vancouver, B.C., Canada V6T 1W5.
† Permanent address: Department of Physiology, University of Illinois, Urbana 61801.
‡ Permanent address: Department of Zoology, University of Washington, Seattle 98105.

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Thymic Muscle Cells Bear Acetylcholine Receptors: Possible Relation to Myasthenia Gravis

Abstract. *Culture of dissociated thymus from rats and humans yielded cells identical to skeletal muscle with respect to morphology, contractility, electrophysiological properties, and the presence of acetylcholine receptors. These cells, strategically located in the thymus, may play a role in initiation of the autoimmune response against acetylcholine receptors, which is characteristic of myasthenia gravis.*

The thymus gland has long been suspected of playing an important role in myasthenia gravis (MG), a disorder manifested by weakness and fatigability of muscles. Clinical pathological evidence implicating the thymus in MG includes a high incidence of thymic hyperplasia (approximately 65 percent) and neoplasia (approximately 10 percent) in patients with MG (*1*), and beneficial effects of thymectomy in many patients (*2*). Studies of acetylcholine receptors (AChR) provide further evidence for a possible link between the thymus and MG. At present, the basic abnormality in MG is generally thought to be a reduction of available AChR at neuromuscular junctions (*3*), resulting from an autoimmune attack directed against receptors (*4*, *5*). Extracts of thymic tissue have now been shown to contain AChR (*5*, *6*), suggesting that an autoimmune reaction against AChR might be initiated within the thymus gland itself.

The source of AChR in the thymus is not yet known. In our study, we have considered the possibility that the AChR might be situated on "myoid" or musclelike cells, first described in the thymus in 1905 (*7*). Such cells are difficult to study in situ, because they are embedded within the tissue mass of the thymus gland. We have therefore used tissue culture techniques (*8*) to obtain sufficient quantities of intact cells for characterization of certain of their physiological, pharmacological, and morphological properties. The results indicate that muscle cultured from human or rat thymus is a rich source of AChR; further, thymic muscle corresponds to cultured skeletal muscle derived from more conventional sites of origin in all properties tested.

Thymuses were dissected from 8-week-old Sprague-Dawley rats, with care being taken to ensure that no tissue from the adjacent areas was included. For each experiment six thymuses were minced, trypsinized, and mechanically dissociated. The thymic cells were plated on plastic cover slips or in plastic wells and maintained in Dulbecco's modified Eagle's medium (*9*) supplemented

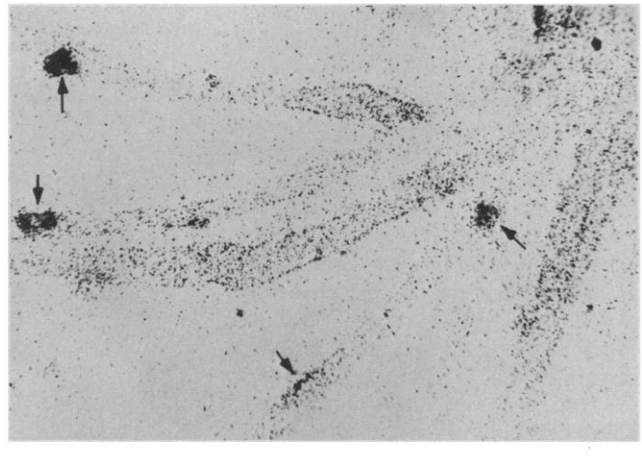

Fig. 1. Autoradiogram of rat thymic myotubes in cell culture after incubation with ^{125}I-labeled α-bungarotoxin. Grains are present over myotubes, an indication of surface acetylcholine receptors. The arrows mark hot spots, or areas of high AChR concentration.