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ON THE ROLE OF FUMARATE REDUCTASE IN ANAEROBIC CARBOHYDRATE CATABOLISM OF *MYTILUS EDULIS* L.

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Abstract—1. The role of the fumarate:NADH oxidoreduction in the anaerobic glycolysis of the sea mussel is examined and discussed.

2. Fumarate reductase activity is present in submitochondrial particles especially from adductor muscle, digestive gland and mantle.

3. The pH optimum of the enzyme complex is 7.9; the approx K_m's for NADH and fumarate are 4.0×10^{-5} M and 6.3×10^{-5} M, respectively.

4. The enzyme complex is inhibitied by amytal, antimycin, ethanol, malonate, phosphate, rotenone, and succinate, and stimulated by Mg^{2+}.

5. It is concluded that part of the mitochondrial respiratory chain is involved in the reduction of fumarate by NADH, comprising site 1 of the oxidative phosphorylation.

INTRODUCTION

The littoral bivalve *Mytilus edulis* displays a dual metabolic strategy in order to survive anoxia. First, as a consequence of slowing down various physiological functions, there will be a strong reduction in energy demand. Secondly, the animal has realized a mode of anaerobic fuel utilization which results in a comparatively high ATP yield per glycosyl unit.

Concerning the first aspect, De Zwaan & Wijsman (1976) calculated that, based on a carbohydrate-dependent energy metabolism, there would be a decrease in energy demand by a factor of 18.5. They compared the ATP equivalents of the oxygen consumption at 13°C in well-aerated seawater with those of the accumulated anaerobic end products and the alterations in the ATP and phosphoarginine pools after 48 hr of anoxybiosis at 13°C in the whole animal. A similar reduction of the metabolic rate has been found for the mussel *Modiolus demissus* by Pamatmat (1979), who applied a direct calorimetric method. This author observed that the anaerobic heat production at 20°C was 7.5% of the aerobic heat production. Ebberink *et al.* (1979) investigated the energy expenditure of the adductor muscle as a function of duration of shell valve closure, and they found a gradual drop until a minimum value was reached of about 20% of that on the onset.

Concerning the second aspect, the formation of succinate and propionate as main products of anaerobic glycolysis is in respect of the output of ATP more economical than the carbohydrate degradation to lactate, as occurs in skeletal muscle of vertebrates, and in crustaceans during anoxia and vigorous activity (De Zwaan & Skjoldal, 1978; Burke, 1979), or to octopine [N-(1-carboxyethyl)-arginine], as occurs in mantle muscle of cephalopods or the central adductor of scallops during or at recovery of the swimming escape response (see Zandee *et al.*, 1980). Formation of succinate and propionate leads to an ATP yield of 13 and 17%, whereas formation of lactate, alanine or octopine results in an ATP yield of about 8% of complete oxidation (3 and 37 equivalents of ATP per glycosyl unit, respectively).

Ebberink *et al.* (1979) have shown that the implication of the combined effect of reduction in energy demand and improvement of ATP output of the anaerobic carbohydrate degradation may be a moderate increase of glycolytic flux during the first few hours following valve closure, but within a few hours the flux has reduced to a value approaching that of the lowest possible flux during aerobic metabolism.

The regulatory aspects of anaerobic metabolism of *M. edulis* concern questions as to which enzymes or enzyme systems in which cell compartments are activated or inhibited in such a way that, with maintenance of an almost constant flux, glycogen is converted to succinate and propionate and to a lesser extent to alanine.

The past years it has been proven that the conversion of phosphoenolpyruvate (PEP) is an important regulatory site. Two enzymes compete for the catabolic conversion of PEP: pyruvate kinase (PyK) and phosphoenolpyruvate carboxykinase (PEPCK). The former appears to belong to the allosteric or regulatory type of enzymes, and its activity is modulated by the concentration of H^+, alanine and fructose-1,6-diphosphate in such a way that it decreases during anoxia (De Zwaan & Holwerda, 1972; Holwerda & De Zwaan, 1973). The latter enzyme has under aerobic conditions a much lower activity than PyK, but is activated during anoxia by changes in the concentration of H^+ and PEP (De Zwaan & De Bont, 1975; Ebberink & De Zwaan, 1980).

Crossover plots of glycolytic intermediates at various stages of anoxybiosis (Ebberink, 1979; Ebberink & De Zwaan, 1980) make clear that the glycolytic flux after valve closure is controlled initially by phosphofructokinase, and that within four hours the two enzymes participating in the PEP branch point take over this control.

The switch at the branch point in converting PEP

away from pyruvate and towards oxaloacetate will be realized gradually in accordance with the increasing strength of the signals that inhibit PyK and stimulate PEPCK. Nevertheless, accumulation of succinate starts straight away from the onset of valve closure. We therefore assume that the initial precursor of succinate is aspartate. This amino acid is converted to oxaloacetate by transamination, the amino group being transferred to pyruvate, which is formed by glycolysis via PyK. Ebberink *et al.* (1979) showed that aspartate is utilized, and, concomitantly, alanine and succinate accumulate. Also for the oyster (Collicutt & Hochachka, 1977) and the lugworm (Felbeck, 1979) it has been found that degradation of glycogen is linked with utilization of aspartate. In a later stage of anaerobic metabolism, when the PEP → pyruvate route is blocked for the major part, glycogen will be the precursor of the accumulating succinate. The two phases of anaerobic glycolysis are represented in Fig. 1a and b.

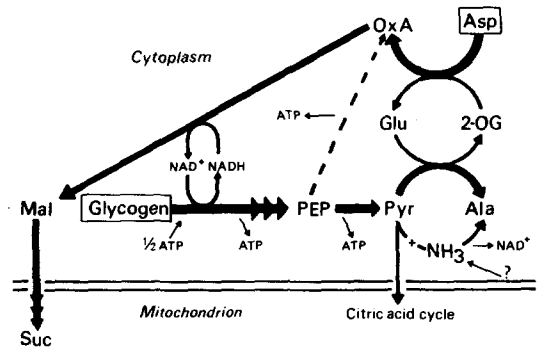

Fig. 1a. Scheme of the anaerobic carbohydrate catabolism in the initial phase.

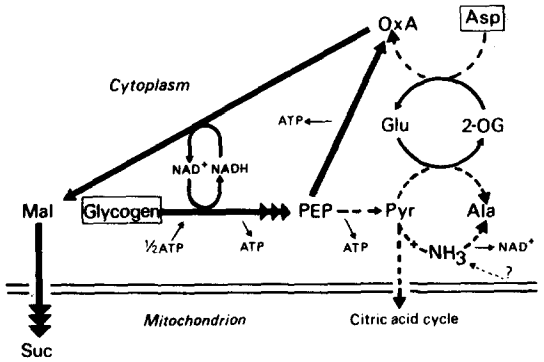

Fig. 1b. Scheme of the anaerobic carbohydrate catabolism after some hours.

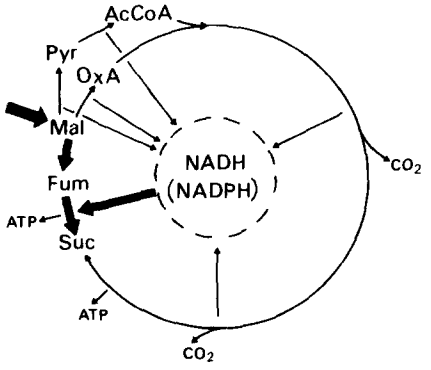

Fig. 1c. Scheme of the mitochondrial part of the anaerobic carbohydrate catabolism.

The formation of malate from oxaloacetate balances the redox state in the cytoplasmic part of the cell. At this stage the cytoplasmic glycolysis is finished. Malate dehydrogenase has taken over the role of lactate dehydrogenase and of octopine dehydrogenase in balancing the oxidation of glyceraldehyde phosphate in other anaerobically functioning systems.

The next part of the anaerobic glycolysis occurs within the mitochondrion after malate has passed the mitochondrial membranes (Fig. 1c). It is in this part that the gain in ATP yield as compared to formation of lactate or octopine is realized. The key reaction is the reduction of fumarate to succinate by way of the so-called fumarate reductase complex. Theoretically the reaction can include part of the mitochondrial electron transport chain (ETC) if the reducing equivalents are donated by the pyridine nucleotides NADH or NADPH. In this part of the ETC site 1 of the oxidative phosphorylation is comprised, which means that one equivalent of ATP can be formed for each equivalent of fumarate reduced. The reducing equivalents originate from the conversion of part of the malate in the citric acid cycle in clockwise direction up to the stage of succinate. The reactions catalyzed by malate dehydrogenase, malic enzyme (via NADPH), pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase are the source of reduced NAD. In a final stage, starting after about 16 hr of anoxia, succinate is converted to propionate. This reaction is, theoretically, accompanied by a substrate-level phosphorylation of ADP.

The NADH:fumarate oxidoreduction has been established for various obligatorily and facultatively anaerobic bacteria and parasitic worms (for references, see Zandee *et al.*, 1980). Schroff & Schöttler (1977) and Schöttler (1977) investigated the ATP yielding fumarate reduction in the euryoxic, free living worms *Arenicola marina* and *Tubifex* sp. We ourselves showed that mussel mitochondria *in vitro* metabolize malate to succinate, accompanied by oxidative phosphorylation (Holwerda & De Zwaan, 1979).

In the fumarate reductase reaction fumarate replaces oxygen as the terminal electron acceptor. Zs.-Nagy (1977) has claimed the presence of yet another type of terminal electron acceptor. Various tissues of molluscs, especially central nervous tissue, contain yellow pigment granula. The pigment is a lipochrome containing unsaturated fatty acids, which are believed to act as electron acceptors with a redox potential exceeding that of cytochrome oxidase. Theoretically at least two phosphorylation sites can be passed. It has been observed that the morphological appearance of the lipid droplets in the granula changes strongly during anoxia. Moreover, Zs.-Nagy found a correlation between the degree of pigmentation of the nervous system and the tolerance of species of anoxia.

In this paper we will especially deal with the anaerobic electron transfer in submitochondrial particles, isolated from mantle of *M. edulis*, as well as from

some other organs. Particularly, the kinetics and mechanism of the NADH:fumarate oxidoreductase complex will be described.

MATERIALS AND METHODS

Mussels were collected in the Dutch Wadden Sea, and held in the laboratory in circulating and filtered seawater at 13°C for some weeks. Mitochondria from different organs except adductor muscle and foot were prepared as described for mantle tissue (Holwerda & De Zwaan, 1979). Adductor muscle and foot were homogenized in 2 vol (v/w) homogenization buffer with a Sorvall Omnimixer (up to maximal speed and down in 20 sec). Then 7 vol of buffer were added to the suspension, and foot tissue was further homogenized using an Ultraturrax homogenizer at half-maximum speed for 2 × 5 sec periods with intervals of 30 sec. The further preparation of mitochondria was as for mantle. Mitochondria were disrupted by sonication with a Branson B-12 Sonifier in a hypotonic medium (25 mM Hepes buffer, pH 7.5) for 15 × 5 sec with intervals of 10 sec, the vessel being cooled in ice water. Power output was 120 W (sonication weight 2.0 g). The resulting submitochondrial suspension was centrifuged at 48,000 *g* for 30 min. The sediment was resuspended in buffer solution and the suspension centrifuged again at 48,000 *g* for 30 min. This method has been optimized with respect to the sonication and centrifugation procedure to attain maximal NADH oxidase activity per unit wet weight, and minimal contamination with glutamate + malate oxidizing activity and malate dehydrogenase activity. Oxygen consumption rates of the sonicated fraction before and after centrifugation are listed in Table 1.

Fumarate reductase activity was determined by following the decrease of NADH concentration (ΔE_{340}) on incubation of submitochondrial particles (SP) with fumarate, either *in vacuo* in Thunberg tubes or under air in the presence of cyanide, to prevent the NADH oxidase reaction. In the first case the tubes were subjected twice to a cycle of evacuation for 20 min down to a pressure below 1 mm Hg and reaeration with N_2. The reaction medium consisted of 2.1 ml 25 mM Hepes buffer, pH 7.5 (or variable when necessary) and 0.25 ml SP-suspension (equivalent to 1.0 g wet weight, unless stated otherwise). After preincubation for 15 min at 25°C the reaction was started by the addition of 0.05 ml NADH solution, and 0.05 ml Na–fumarate solution or water for the blank. In the cyanide poisoned system KCN solution, previously adjusted to the right pH, was added in a final concentration of 6 mM. Incubation was carried out at 25°C for periods ranging from 15 min to 1 hr. The reaction was stopped by putting the tubes into ice water, followed by centrifugation in the cold at 48,000 *g* for 10 min. Extinction at 340 nm of the supernatant was measured immediately. Occasionally, the supernatant was fractionated on Dowex 1 × 8 for the enzymatic and isotachophoretic determination of fumarate, malate and succinate, as described earlier (Holwerda & De Zwaan, 1979). For evaluation of kinetic measurements the modification by Lee & Wilson (1971) of the Lineweaver–Burk expression was applied:

$$1/\bar{v} = 1/V + K_m/V \cdot 1/\bar{S},$$

with

$$\bar{v} \equiv (S_0 - S)/t$$

and

$$\bar{S} \equiv (S_0 + S)/2.$$

Inhibition curves were composed of $1/\bar{v}$ vs [inhibitor], which theoretically is a linear relationship.

Succinate dehydrogenase activity was determined essentially according to Ackrell *et al.* (1978). Conditions had been optimized with respect to PMS concentration (0.44 mM) and preincubation of SP (30 min at 25°C). The reaction was carried out at 25°C, and SDH activity was measured from ΔE_{600} during the first 15–20 sec after addition of dyes and rapid mixing.

Protein concentration was measured with the Coomassie brilliant blue method (Bradford, 1976), with bovine serum albumin as the reference protein.

RESULTS

The reaction rate (NADH utilization) of the reaction—fumarate + NADH → succinate + NAD^+—has been determined in a system either in the presence of 6 mM KCN or *in vacuo* (Table 2). It appears that the rate is essentially similar under either condition. In the case of KCN, the rate was determined at two incubation times. The result indicates that the rate slows down when the incubation time progresses.

In order to check the stoichiometry of the reaction, both NADH utilization and succinate production were compared in a submitochondrial fraction of mantle tissue (Table 3). The production of succinate clearly exceeds the utilization of NADH, which means that the former is not an exact parameter of the oxidation of NADH by fumarate catalyzed by submitochondrial particles. Apparently, there is an additional reduction of fumarate, which is not accompanied by conversion of added reduced coenzyme. As the mitochondrial inner membrane is impermeable to NADH, only the change in this coenzyme concentration represents the submitochondrial fumarate reductase (FR) activity examined for.

Five organs have been examined for the presence of FR activity. Table 4 presents the utilization of NADH in the presence and absence of added fumarate. The difference represents the FR activity. On a weight basis it is the highest in the mantle and the lowest in

Table 1. Oxygen consumption rates of the sonicated fraction before centrifugation (A), and the resuspended 48,000 *g* sediment (B). No detectable oxygen consumption was present in the 48,000 *g* supernatant. Values are in μl $O_2 \cdot hr^{-1} \cdot g^{-1}$ wet wt

Substrate (mM)	Oxygen consumption A	B
None	4	0
NADH (6)	150	109
Succinate (6)	47	37
Malate (6) + glutamate (6)	20	4
Ibid. + NAD^+ (6)	114	32

Table 2. Comparison of the reaction rate (utilization of NADH) determined in the presence of 6 mM KCN or *in vacuo*. Conditions: 0.2 mM NADH, 1 mM fumarate. Values are in nmol $NADH \cdot hr^{-1} \cdot g^{-1}$ wet wt (mantle tissue), corrected for the blank (without fumarate); $n = 4$, ± SD

Incubation with KCN		*in vacuo*
15 min	30 min	30 min
182 ± 4	153 ± 3	156 ± 5

Table 3. Comparison of the utilization of NADH and the production of succinate (enzymatic and isotachophoretic determination) in the FR reaction. Conditions: 0.2 mM NADH, 1 mM fumarate; incubation time 40 min, in the presence of 6 mM CN^-. Values are in nmol·hr^{-1}·g^{-1} wet wt, corrected for the blank (without fumarate); $n = 2$

NADH used	Succinate produced (enzymat.)	Succinate produced (isotachoph.)
126	166	158
144	168	185

Table 4. Anaerobic oxidation of NADH in five organs (submitochondrial particles) in the presence and absence of fumarate. Conditions: 0.2 mM NADH, 1 mM fumarate; incubation time 30 min *in vacuo*. Values are in nmol NADH·hr^{-1}·g^{-1} wet wt; $n = 3$

	Fumarate added	No fumarate (blank)	Net reaction
Adductor muscle	121	14	107
Gill	16	8.4	7.6
Foot	84	5.7	78
Digestive gland	132	1.2	131
Mantle	170	1.2	169

the gill. In the absence of any added oxidizer there is only a marginal activity.

The activity of FR is low in case of NADPH as the reductor (Table 5). When also NAD^+ is added, an activity equal to that in the presence of an equimolar amount of NADH is obtained. So, indirectly NADPH equals the ability of NADH to serve as an electron donor. The pH optimum of the particulate FR is 7.9–8.0 (Fig. 2). This value differs from the optimum pH of 7.2 at which intact mitochondria metabolize malate to succinate (Holwerda & De Zwaan, 1979).

In Figs 3 and 4 Lineweaver–Burk plots are presented for NADH and fumarate, respectively. The apparent K_m values are 4.0×10^{-5} M and 6.3×10^{-5} M, respectively. The value of V_{max} was

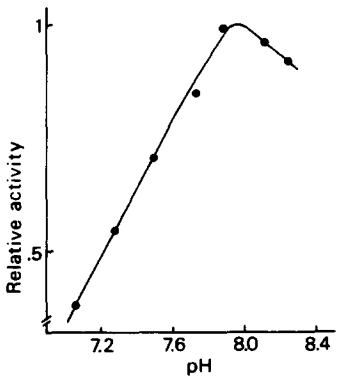

Fig. 2. pH dependence of fumarate reductase activity. Conditions: 40 mM Hepes buffer; 0.5 mM NADH; 1 mM fumarate; cyanide present; incubation time 25 min; pH values measured after the reaction. Each point is the mean of three incubations.

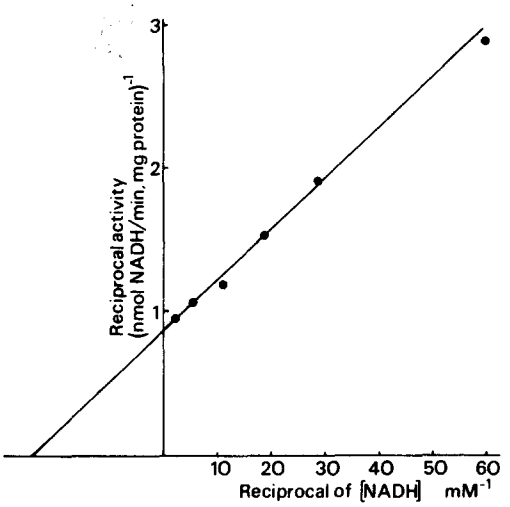

Fig. 3. Lee–Wilson plot ($1|\bar{v}$ vs $1|\bar{S}$) of fumarate reductase activity vs concentration of NADH. Conditions: 3 mM fumarate; cyanide present; incubation time 15 min. Each point is the mean of two incubations.

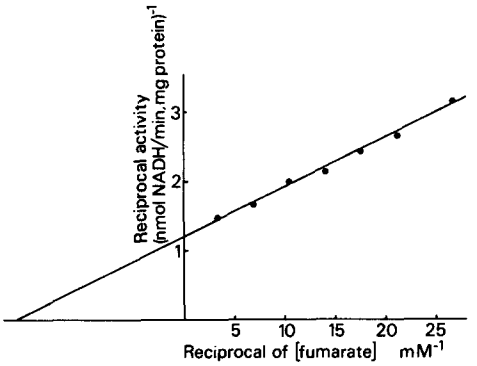

Fig. 4. Lee-Wilson plot ($1|\bar{v}$ vs $1|\bar{S}$) of fumarate reductase activity vs concentration of fumarate. Conditions: 0.2 mM NADH; cyanide present; incubation time 15 min. Each point is the mean of two incubations.

within the range of 0.9–1.1 nmol NADH. (min, mg protein)$^{-1}$.

The effect of the uncoupler *p*-CF_3O-CCP and of $MgCl_2$, HPO_4^{2-} and ADP is shown in Table 6. The submitochondrial particles (SP) appear to be uncoupled as the uncoupler does not stimulate the reac-

Table 5. Comparison of NADH, NADPH, and NADPH + NAD^+ as electron donor for fumarate reductase. Conditions: 1 mM fumarate, incubation time 15 min, in the presence of CN^-. All values (nmol NAD(P)H·hr^{-1}·g^{-1} wet mantle tissue) are the mean of two incubations

0.05 mM NADH	258
0.05 mM NADPH	29
Ibid. + 0.05 mM NAD^+	251
0.10 mM NADPH	47
Ibid. + 0.05 mM NAD^+	256
0.20 mM NADPH	50
Ibid. + 0.05 mM NAD^+	238

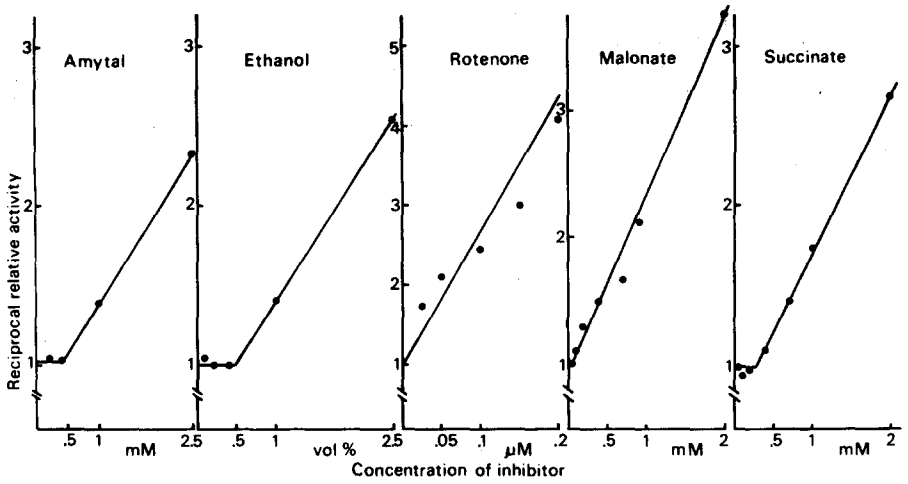

Fig. 5. The effect of electron transfer chain inhibiting agents and succinate on fumarate reductase activity. Conditions: 0.2 mM NADH, 1.0 mM fumarate; cyanide present; incubation time 15 min. Rotenone was added in a non-inhibitory volume of ethanol.

tion. Mg^{2+} stimulates weakly and acts as an antagonist of the inhibitory effect by phosphate and ADP.

For mantle SDH an apparent K_m for succinate of 1.0×10^{-4} M was estimated. Even though this value is higher than that of FR for fumarate, it is fairly low. For the flight muscle of the locust *Locusta migratoria*, an obligatory aerobic organ, we have determined a higher value of the K_m of SDH for succinate, being 6×10^{-4} M.

In Fig. 5 and Table 7 the effect of inhibitors of the respiratory chain on the FR activity is shown. Inhibitors of the mammalian NADH dehydrogenase complex (NADH–ubiquinone oxidoreductase) and the succinate dehydrogenase complex (succinate–ubiquinone oxidoreductase) also inhibit fumarate reductase. The $I_{0.5}$ values for malonate and succinate are 0.8 mM and 1.3 mM, respectively. The K_i values of FR for malonate and succinate cannot be calculated as the inhibited reaction step (SDH → fumarate) apparently is not rate limiting. Antimycin A only inhibits at relatively high concentration. The same phenomenon was observed for FR activity in SP from beef heart (Sanadi & Fluharty, 1963), and from *Tubifex* sp. musculature (Schöttler, 1977). A similar effect of antimycin A has been found on SDH activity in mantle SP (Table 7).

Table 6. The effect of *p*-CF_3O-CCP, Mg^{2+}, phosphate and ADP on FR activity. Conditions: 0.2 mM NADH, 1 mM fumarate, incubation time 15 min, in the presence of CN^-. Values are in nmol $NADH \cdot hr^{-1} \cdot g^{-1}$ wet wt; $n = 4$, ± SD

Addition (mM)	Enzyme activity
None	155 ± 5
p-CF_3O-CCP (10^{-3})	150 ± 7
Mg^{2+} (5)	176 ± 4
P_i (1)	92 ± 8
Mg^{2+} (5) + P_i (1)	128 ± 12
P_i (1) + ADP (0.2)	62 ± 8
Mg^{2+} (5) + P_i (1) + ADP (0.2)	160 ± 8

Table 7. The effect of antimycine A on reactions of submitochondrial particles involving (part of) the respiratory chain. (A) FR of mantle. Conditions: 0.2 mM NADH, 1 mM fumarate; incubation time 15 min, in the presence of CN^-. Values are the mean of two incubations. (B) SDH of mantle (1 mM succinate). (C) FR of *Tubifex* musculature. (D) FR of beef heart. (E) NADH oxidase of beef heart

Addition	Enzyme activity (% of the control) A	B	C	D	E
None	100	100	100	100	100
Ethanol (solvent vol)	102	124			
Antimycin A (μM)					
0.1	98				50
0.5			92	80	~0
1	103	125		60	
2				20	
5			79		
10	81	107			
100	42	72			

DISCUSSION

Of the five organs studied, the mantle exhibits the highest fumarate reductase activity. This outcome is supported by our observation on electron micrographs of tissue slides that the mantle together with the digestive gland show relatively high mitochondrial densities. The extreme low FR activity of the gill is also reflected by the low *in vivo* succinate accumulation in comparison with the other organs (Table 8).

Table 8. Changes in succinate concentration after 24-hr aerial exposure at 13°C. Values are in $\mu mol \cdot g^{-1}$ wet wt (after De Zwaan, 1977)

	Control	Exposed
Adductor muscle	0.12	3.45
Gill	0.03	0.91
Digestive gland	0.19	5.31
Mantle	0.19	2.40
Residu	0.10	2.54

Table 9. Specific enzyme activity (in $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein at 25°C) of PEP carboxykinase in some organs (after De Zwaan, 1977)

Adductor muscle	0.174
Gill	0.009
Foot	0.029
Digestive gland	0.043
Mantle	0.030

However, even when a close relationship between FR activity and succinate production exists: in this type of experiment it may not be observed as the latter compound could be redistributed among the tissues or excreted by the gills to the environment. Arguments against such interferences are the very low succinate concentration in the hemolymph during anoxia and the absence of a significant release of succinate (and propionate) during a period of 12 hr incubation in oxygen-free sea water (Zurburg, personal communication). For the gill also the lowest specific activity of PEP carboxykinase was found (Table 9). On the other hand, its activity in the adductor muscle appears to be relatively high. A certain degree of organ specificity therefore may exist: a main contribution of the adductor muscle to convert glycogen to malate, redistribution of malate, and a relatively high capacity of the mantle and the digestive gland to convert malate to succinate. When also a significant redistribution of succinate should occur, then especially the digestive gland seems the appropriate organ for storage (see Table 8). The significance for this might be found in a relatively high potential of the digestive gland to metabolize succinate during a subsequent aerobic recovery.

The NADH oxidizing capacity in the absence of added fumarate (Table 4) is low. If an endogenous oxidizer, such as the lipochrome pigment as suggested by Zs.-Nagy (1977), would be present in the sea mussel, it is not likely to be found in the mitochondrial fraction. The so-called liposomes, either intact or disrupted, probably will possess a relatively low density and therefore would have been discarded during the isolation procedure of the mitochondrial particles.

Besides NADH also NADPH can donate reducing equivalents in the FR reaction (Table 5). The maximum activity is 20% of that obtained with an equimolar amount of NADH. Probably, endogenous NAD^+ in the particulate preparation accounts for this activity. After addition of NAD^+, NADH can be replaced by NADPH without any change in the enzyme activity, which points to the presence of a transhydrogenase. This also enables the NADP-dependent malic enzyme reaction to provide the reducing equivalents. We have found a NADP-dependent malic enzyme in the mantle of the sea mussel (Freund, De Zwaan & Holwerda, unpublished).

Although the pH optimum of the anaerobic production of succinate from added malate by intact mitochondria at 25°C is 7.2 (Holwerda & De Zwaan, 1979) the value of 7.9 to 8.0 obtained for submitochondrial FR is nearer to that of solubilized SDH from adductor muscle (Ryan & King, 1962), mammalian (Singer *et al.*, 1956) or yeast enzymes (Singer *et al.*, 1957), being 7.7, 7.6 and 7.8, respectively.

The apparent K_m value of FR for NADH is low: 4.0×10^{-5} M compared to 1.0–1.1 $\times 10^{-4}$ M for beef heart and liver NADH dehydrogenase. Also the Michaelis constant for fumarate—6.3×10^{-5} M—is low as compared with the value for fumarate of the homogenous beef heart SDH, being 5.0×10^{-4} M. Similarly low values have been reported for submitochondrial FR of *Arenicola marina* (Schroff & Schöttler, 1977) and *Tubifex* sp. (Schöttler, 1977): 2.5×10^{-5} M and 2.7×10^{-5} M, respectively. These low values allow low levels of fumarate to be reduced by low levels of NADH. We consider this an adaptation of the succinate dehydrogenase/fumarate reductase to facultatively anaerobic metabolism. On the other hand, the K_m of mantle SDH for succinate is not particularly high: 1.0×10^{-4} M as compared with a value of at least 1 mM for solubilized succinate dehydrogenase, for example 1.3 mM in case of beef heart (Singer, 1966) and 2 mM for adductor muscle of *M. edulis* (Ryan & King, 1962). Obviously, when oxygen supply is sufficient the succinate-ubiquinone oxidoreductase exhibits its usual function in the citric acid cycle. For this reason the adaptation of this enzyme complex to an efficient fumarate reduction will be restricted by the need of sufficient affinity of succinate in the succinate oxidation. The value of 1.0×10^{-4} is even smaller than the K_m for succinate of SDH of the flight muscle of *L. migratoria*, in spite of its strictly aerobic energy metabolism. The ratio of K_m (suc): K_m(fum) was found to be 1.58, comparable with a ratio of 1.33 found by Hammen (1975) for the adductor muscle of *M. edulis*. However, this author gives K_m values that are substantially higher than ours: 30 mM for fumarate (at about 20°C in the fumarate–NADH assay), and 40 mM for succinate (in the succinate–PMS assay). It is not likely that such a high apparent K_m for fumarate may allow any significant *in vivo* activity for fumarate reductase.

The submitochondrial particles show no coupling as neither the uncoupler *p*-CF_3O-CCP, nor ADP + P_i enhances the FR activity. It is remarkable that phosphate has an inhibitory effect, which is reinforced by ADP. This inhibition is counteracted by the addition of Mg^{2+}. Solely added, Mg^{2+} stimulates the enzyme slightly. In contrast with these results, it is known that phosphate stimulates the reverse reaction by activating SDH. Our data indicate that there will be a need of fumarate reductase for Mg^{2+}.

The inhibition of FR by amytal, ethanol, malonate, rotenone and succinate proves that the reaction proceeds through part of the mitochondrial respiratory chain, namely the complexes NADH–ubiquinone oxidoreductase and ubiquinone–fumarate oxidoreductase. There are no indications that the latter differs from complex II, the succinate–ubiquinone oxidoreductase. For *Tubifex* sp. Schöttler (1977) suggested that in the second part of the electron transfer chain cytochrome b could be involved. We also observed that antimycin A, which inhibits the transfer at the level of cytochrome b, inhibits the reduction of fumarate. However, this inhibition is only displayed at relatively high concentrations of antimycin A (Table 7), and is also observed in the succinate → PMS → DCIP reaction at about the same concentrations of

inhibitor. The inhibition in fact could be due to the presence of a cytochrome of the *b*-type (Davis *et al.*, 1972) in the succinate dehydrogenase complex. Possibly, this cytochrome possesses a lower affinity for the inhibitor than the cytochrome *b* of complex III.

As phosphorylation site I is linked to the electron transfer of NADH to ubiquinone, it can be expected that the fumarate reductase reaction is coupled to the phosphorylation of one equivalent of ADP. Indeed, we have proven (Holwerda & De Zwaan, 1979) that intact mitochondria convert malate to succinate with simultaneous production of ATP with a $P/2e^-$ ratio of 0.46. As is evident from the data in Table 6, sonication of the mitochondria has resulted in uncoupling.

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