Transhydrogenase and the anaerobic mitochondrial metabolism of adult *Hymenolepis diminuta*

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SUMMARY

The adult cestode, *Hymenolepis diminuta*, is essentially anaerobic energetically. Carbohydrate dissimilation results in acetate, lactate and succinate accumulation with succinate being the major end product. Succinate accumulation results from the anaerobic, mitochondrial, 'malic' enzyme-dependent utilization of malate coupled to ATP generation via the electron transport-linked fumarate reductase. A lesser peroxide-forming oxidase is apparent, however, fumarate reduction to succinate predominates even in air. The *H. diminuta* matrix-localized 'malic' enzyme is NADP-specific whereas the inner membrane (IM)-associated electron transport system prefers NADH. This dilemma is circumvented by the mitochondrial, IM-associated NADPH→NAD^+ transhydrogenase in catalyzing hydride ion transfer from NADPH to NAD^+ on the IM matrix surface. Hydride transfer is reversible and phospholipid-dependent. $NADP^+$ reduction occurs as a non energy-linked and energy-linked reaction with the latter requiring electron transport NADH utilization or ATP hydrolysis. With NAD^+ reduction, the cestode transhydrogenase also engages in concomitant proton translocation from the mitochondrial matrix to the intermembrane space and supports net ATP generation. Thus, the cestode NADPH→NAD^+ system can serve not only as a metabolic connector, but an additional anaerobic phosphorylation site. Although its function(s) is unknown, a separate IM-associated NADH→ NAD^+ transhydrogenation, catalyzed by the lipoamide and NADH dehydrogenases, is noted.

Key words: Transhydrogenase, mitochondria, anaerobic energetics, *Hymenolepis diminuta*, cestode, metabolism.

ENERGETIC METABOLISM OF ADULT *HYMENOLEPIS DIMINUTA*

The adult intestinal helminth, *Hymenolepis diminuta*, continues to serve as a model for the study of cestode physiologically anaerobic energetics as well as the anaerobic energetics of parasitic helminths generally. It is now well established that carbohydrate dissimilation by adult *H. diminuta* results in acetate, lactate and succinate accumulation, with succinate being the major end product (Fairbairn *et al*. 1961; Scheibel and Saz, 1966; Watts and Fairbairn, 1974). Equally convincing are the data demonstrating that succinate accumulation is the product of the anaerobic, mitochondrial, ATP-generating utilization of malate (Scheibel and Saz, 1966; Bueding and Saz, 1968; Saz *et al*. 1972).

In *H. diminuta*, malate arises in the cytosol via CO_2 fixation into phosphoenolpyruvate followed by reduction of the resulting oxalacetate (Prescott and Campbell, 1965; Scheibel and Saz, 1966; Bueding and Saz, 1968; Scheibel *et al*. 1968). Cytosolic malate then serves as the mitochondrial substrate. Upon entering the mitochondrial matrix, malate is oxidatively decarboxylated by the NADP-specific 'malic' enzyme, thereby yielding pyruvate, CO_2 and reducing power for electron transport in the form of NADPH (Prescott and Campbell, 1965; Li *et al*. 1972; Saz *et al*. 1972; McKelvey and Fioravanti, 1984, 1985). Malate also is converted to fumarate by matrix fumarase (Read, 1953; McKelvey and Fioravanti, 1985). With the electron transport-coupled reduction of fumarate to succinate, as catalyzed by the fumarate reductase, the malate dismutation reaction is completed (Scheibel and Saz, 1966; Saz *et al*. 1972). Net, anaerobic ATP generation by isolated *H. diminuta* mitochondria is site I-dependent, as indicated by rotenone-sensitivity, and is significantly inhibited by oligomycin and protonophoric agents (including niclosamide, a known anticestodal agent (Saz *et al*. 1972)), but not antimycin A. In addition, phosphorylation by *H. diminuta* mitochondria is inhibited by malonate in keeping with the involvement of the fumarate reductase system (Saz *et al*. 1972).

Aside from the NADH-utilizing fumarate reductase (Scheibel *et al*. 1968), *H. diminuta* mitochondria display a less active, inner membrane-associated, rotenone-sensitive and peroxide-forming NADH oxidase that appears to require tightly bound

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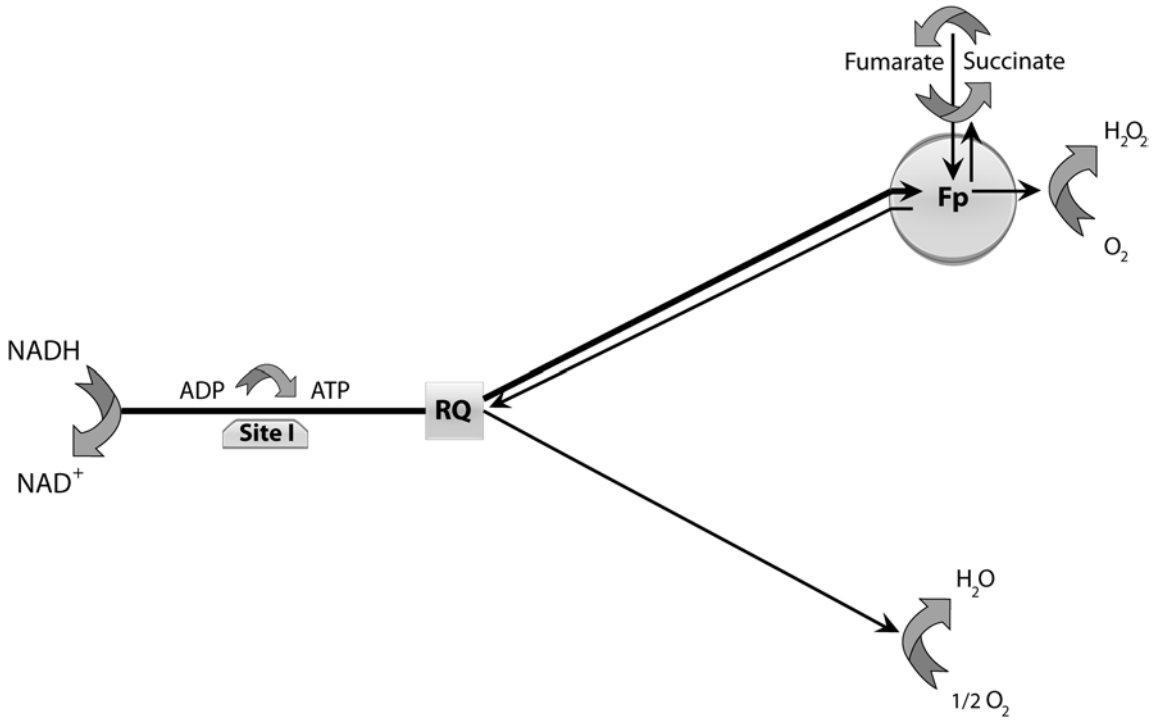

Fig. 1. Model of the *Hymenolepis diminuta* mitochondrial electron transport system. The physiological route for reducing equivalents is indicated by bold lines. Designations are as follows: RQ, rhodoquinone; Fp, flavin-containing component of the fumarate reductase.

manganous ion. Peroxide is formed by NADPH oxidation as well (Fioravanti and Saz, 1980; Fioravanti, 1981, 1982*a*; McKelvey and Fioravanti, 1985; Fioravanti and Reisig, 1990). Both the NADH-utilizing fumarate reductase and oxidase exhibit a benzoquinone preference inasmuch as they employ rhodoquinone rather than ubiquinone as reflected in NADH oxidation, NADH-dependent oxygen consumption, hydrogen peroxide production, and succinate accumulation (Fioravanti and Kim, 1988). *H. diminuta* mitochondria also display a membrane-associated succinoxidase activity that results in oxygen consumption and peroxide formation (Fioravanti, 1982*a*; Fioravanti and Reisig, 1990). NADH- and succinate-dependent peroxide formation accounts for about 50 and 40 percent, respectively, of the total oxygen consumed by isolated mitochondrial membranes (Fioravanti and Reisig, 1990). Interestingly, the fumarate reductase inhibitor, malonate (Saz *et al.* 1972), not only inhibits fumarate reduction and succinate oxidation, but also significantly inhibits *H. diminuta* mitochondrial NADH oxidase (Fioravanti, 1982*a*; Fioravanti and Reisig, 1990). Consistent with the physiological role of fumarate as the terminal electron transport acceptor, NADH utilization by *H. diminuta* mitochondrial membranes increases precipitously in the presence of fumarate (despite the presence of oxygen) whereas NADH- and succinate-dependent peroxide accumulation is virtually abolished and oxygen consumption is minimal. Neither NADH- nor succinate-dependent oxygen utilization by *H. diminuta* mitochondrial membranes are appreciably sensitive to antimycin A, sodium azide or potassium cyanide (Fioravanti and Reisig, 1990).

Taken together, the above findings support the model proposed by Fioravanti and Reisig (1990) of the *H. diminuta* mitochondrial electron transport system given in Fig. 1. Accordingly, both oxygen- and fumarate-dependent NADH oxidations employ a common site I-containing complex, viz., an NADH–rhodoquinone reductase. Branching occurs after this complex, at the benzoquinone level, as indicated by the rhodoquinone requirement of NADH-dependent oxygen utilization and peroxide formation. Because malonate inhibits NADH-/succinate-dependent peroxide formation/oxygen utilization as well as fumarate reduction, it appears that the NADH oxidase, succinoxidase, and fumarate reductase use a common malonate-sensitive, flavin-containing fumarate reductase that terminates the major branch of the system (Fig. 1). This is supported further since the presence of fumarate virtually abolishes NADH-dependent peroxide formation, and succinate oxidation. Succinate-dependent peroxide accumulation/oxygen consumption involves the above-mentioned flavin-containing branch and the lesser branch originating at the rhodoquinone. Thus, reducing power from NADH or succinate uses both branches of the electron transport system to reduce oxygen while fumarate reduction would need only the major physiological branch. The non-peroxide-forming branch is presumed to result in water formation (Fig. 1).

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Adult *H. diminuta* mitochondria display cytochrome c reducing and oxidizing activities, i.e. membrane-associated NAD(P)H- and succinate-dependent cytochrome c reductase, cytochrome c oxidase, and a cytochrome c peroxidase as assessed by exogenous oxidized or reduced cytochrome c utilization (Kim and Fioravanti, 1985; McKelvey and Fioravanti, 1986). Rotenone-sensitive NADH-dependent cytochrome c reductase and succinate-dependent cytochrome c reductase activities exhibit antimycin A sensitivity whereas cytochrome oxidase activity is inhibited by sodium azide and potassium cyanide. In contrast, these inhibitors are without appreciable effect on *H. diminuta* NADH oxidase, succinoxidase and fumarate reductase (Saz *et al.* 1972; Fioravanti and Reisig, 1990). While fumarate reduction involves the major branch of the cestode electron transport system, thereby explaining a lack of significant antimycin A, azide and cyanide effects, at this juncture it is assumed that membrane-associated, exogenous cytochrome c reduction/oxidation reflects activities of the lesser branch of the cestode electron transport system (Fig. 1). Even though further study is needed, the lack of appreciable inhibition of NADH oxidase and succinoxidase by antimycin A, azide and cyanide may reflect a basal level of cytochrome c-dependent activities, under the conditions of assay, that cannot be diminished further by the inhibitors. Nonetheless, these findings and others demonstrating rotenone-sensitive and -insensitive NADH-dependent cytochrome c reductase (McKelvey and Fioravanti, 1985), point to cytochrome c as an interesting component of *H. diminuta* mitochondria.

The *H. diminuta* electron transport system can form peroxide when exposed to oxygen (e.g. Kim and Fioravanti, 1985; Fioravanti and Reisig, 1990). It is noteworthy, therefore, that adult *H. diminuta* lacks appreciable catalase, glutathione peroxidase or NAD(P)H peroxidase activities, but does display peroxide-forming superoxide dismutase activity (Paul and Barrett, 1980; Barrett and Beis, 1982). Although the subject of differing opinions, the occurrence and intramitochondrial localization of peroxidase activity (or peroxidase-like activity) in adult *H. diminuta* has been reported using histochemical and biochemical methods (Threadgold *et al.* 1968; Lumsden *et al.* 1969; Robinson and Bogitsh, 1976, 1978; Paul and Barrett, 1980; Kim and Fioravanti, 1985; McKelvey and Fioravanti, 1986). A mitochondrial membrane association for a *H. diminuta* cytochrome c peroxidase has been indicated (Threadgold *et al.* 1968; Robinson and Bogitsh, 1976, 1978; Paul and Barrett, 1980), but Kim and Fioravanti (1985), using a biochemical assessment found that cytochrome c peroxidase activity is associated predominantly (95% of recovered activity) with the soluble mitochondrial fraction. Indeed, an evaluation of the intramitochondrial localization of the cestode cytochrome c peroxidase indicated a 55% matrix and 32% intermembrane space distribution, respectively (McKelvey and Fioravanti, 1986). Given the potential for peroxide formation by *H. diminuta* mitochondria and the apparent lack of a number of common peroxide-utilizing systems in the cestode, the occurrence of mitochondrial cytochrome c peroxidase activity merits additional study.

Further compelling data supporting the physiologically anaerobic character of *H. diminuta* energetics are evident in terms of *in vitro* cultivation. Schiller (1965) demonstrated that *H. diminuta* can be cultivated from excysted cycticercoid to ovigerous adult in an atmosphere of 97% N_2-3% CO_2. Moreover, Schiller demonstrated that oncospheres, derived from these cultivated cestodes, develop into infective cysticercoids in the intermediate beetle host. Employing cultivation methods (modified by Schiller), Roberts and Mong (1969) cultivated 6-day-old *H. diminuta* for 12 days in an atmosphere of 95% N_2-5% CO_2 with egg production; these eggs developed into infective cysticercoids when given to beetles. The addition of O_2 to the gas phase (1, 5, 20%) did not stimulate helminth growth. Voge *et al.* (1976) found that *in vitro* cultivation of hatched *H. diminuta* oncospheres to infective cysticercoids occurred more rapidly in 100% N_2 than in air. Of note were the findings that use of 95% N_2-5% CO_2 or 95% N_2-5% O_2 atmospheres gave results similar to those with 100% N_2, thereby indicating that CO_2, while needed for post-cycticercoid development, is not essential to cysticercoid development itself.

Saz *et al.* (1972) were the first to note that NADPH, formed by the action of the 'malic' enzyme, could serve as a source of reducing power for NADH-dependent, anaerobic phosphorylation in adult *H.diminuta* mitochondria. More importantly, their data indicated that the intermediary action of a membrane-associated NADPH→NAD^+ transhydrogenase in *H. diminuta*, and presumably other helminth systems, plays a crucial role in anaerobic, electron transport-coupled succinate formation. Thus, for the first time in any of the invertebrates, a physiological role of the transhydrogenase as a metabolic connector was made apparent in the *H. diminuta* model.

THE *HYMENOLEPIS DIMINUTA* MITOCHONDRIAL NADPH→NAD^+ SYSTEM

The *H. diminuta* mitochondrial inner membrane-associated NADPH→NAD^+ transhydrogenase (EC 1.6.1.1) catalyzes a reversible hydride ion transfer as given in the following equation:

$$NADPH + NAD^+ \leftrightarrow NADP^+ + NADH.$$

The NADH- and NADPH-forming activities, denoted as the NADPH→NAD^+ and NADH→$NADP^+$ reactions, respectively, are readily assessed

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Table 1. NADPH Utilization by *Hymenolepis diminuta* mitochondrial membranes under conditions of reduced oxygen tension.

Reaction	Activity	
	Aerobic	Reduced Oxygen Tension
NADPH oxidase	5·5	1·3
NADPH: fumarate	5·5	3·7
NADPH: NAD^+: oxygen	11·2	1·6
NADPH: NAD^+: fumarate	12·8	8·5

Activity expressed as nmoles/min per mg protein; 0·35 mg of protein employed for assay.

spectrophotometrically in the cestode using the appropriate acetylpyridine derivatives of $NAD(P)^+$ (i.e. AcPyAD(P)) as hydride ion acceptors (Saz *et al.* 1972; Fioravanti and Saz, 1976; McKelvey and Fioravanti, 1985). In support of the considerations of Saz *et al.* (1972), a reduced pyridine nucleotide preference of the *H. diminuta* electron transport system is apparent (Fioravanti, 1981) with NADH rather than NADPH being the preferred reductant (Table 1). Significantly, however, pyridine nucleotide specificity is circumvented by the transhydrogenase in its catalysis of NADPH→NAD^+ hydride ion transfer. Whereas NADPH, formed by the 'malic' enzyme, does not appear to be an effective reductant for the oxidase or fumarate reductase, significant rotenone-sensitive NADPH oxidation occurs in the presence of NAD^+. With respect to fumarate-dependent succinate formation, the coupling of the NADPH→NAD^+ transhydrogenase with fumarate reduction by *H. diminuta* mitochondrial membranes is essentially unchanged whether assessed under aerobic conditions or conditions of reduced oxygen tension (Fioravanti, 1981) (Table 1). Additionally, coupling of malate utilization with the *H. diminuta* electron transport system, via the NADPH→NAD^+ transhydrogenase, was demonstrated (McKelvey and Fioravanti, 1984).

An association of the *H. diminuta* transhydrogenase with the mitochondrial membrane fraction was reported by Saz *et al.* (1972) and, subsequently, the transhydrogenase was found to be a mitochondrial inner membrane (IM) component (Fioravanti and Saz, 1976; McKelvey and Fioravanti, 1985). This IM association was demonstrated using hypotonically shocked and sonicated cestode mitochondria, in conjunction with sucrose step-gradient centrifugation and appropriate marker enzymes (Table 2). The transhydrogenase activity was predominantly localized with the *H. diminuta* IM mitochondrial fraction as noted for the IM systems/markers (i.e. NADH oxidase, fumarate reductase, succinate dehydrogenase, and rotenone-sensitive NADH→cytochrome c reductase) (Table 2). The validity of marker enzyme localizations was established employing isolated rat liver mitochondria subjected to the same procedures. The findings with *H. diminuta* mitochondria were supported further by transhydrogenase and marker enzyme assessments following fractionation of incubated cestode mitochondria using increasing amounts of digititonin (McKelvey and Fioravanti, 1985). Assessments of enzyme activities of 'intact' *vs* sonically disrupted *H. diminuta* mitochondria and of isolated submitochondrial particles (SMP) were consistent with transhydrogenase-catalyzed hydride ion transfer, between reduced and oxidized pyridine nucleotides, occurring on the matrix surface of the IM (McKelvey and Fioravanti, 1985; Fioravanti *et al.* 1992). Significantly, the 'malic' enzyme (as well as fumarase) is predominantly localized in the mitochondrial matrix of *H. diminuta*, in accord with the localizations of the intermembrane and matrix markers, i.e. adenylate kinase and citrate synthase (Table 2). Therefore, NADPH reducing power for electron transport would be readily available for transhydrogenation (McKelvey and Fioravanti, 1985).

CHARACTERIZATION OF THE *HYMENOLEPIS DIMINUTA* MITOCHONDRIAL NADPH→NAD^+ TRANSHYDROGENASE

The *H. diminuta* mitochondrial NADPH→NAD^+ transhydrogenase is phospholipid-dependent (Fioravanti and Kim, 1983). When extracted with hexane, lyophilized cestode mitochondrial membranes display increased NADPH→NAD^+ activity, but decreased NADH oxidase and fumarate reductase activities. However, with the addition of aqueous acetone to lyophilized and hexane-treated membranes, a diminishment in NADPH→NAD^+ activity as well as NADH oxidase and fumarate reductase is noted (Fioravanti and Kim, 1983). These data indicate: (1) neutral lipid(s) are needed by the cestode oxidase and fumarate reductase; and (2) the cestode transhydrogenase, NADH oxidase, and fumarate reductase are phospholipid dependent. Consistent with a phospholipid dependency, phospholipase treatments of *H. diminuta* mitochondrial membranes significantly depress transhydrogenase activity with phospholipase A_2 being more effective in this regard than phospholipase C, thereby implying that conversion of membrane phospholipids to lysophosphatides was more inhibitory than conversion to diacylglycerols. Because transhydrogenase activity of either acetone-extracted lyophilized/hexane-treated membranes or phospholipase-treated membranes was unaffected by phospholipid supplementation, partially phospholipid(s)-depleted (~60%) membranes were prepared by detergent-treatment/ammonium sulfate precipitation, i.e. the 30–55 fraction (Table 3). The need for phospholipid

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Table 2. Enzyme distribution in *Hymenolepis diminuta* mitochondria

Reaction	Inner membrane	Outer membrane	Matrix	Intermembrane space
	Total units[a]			
NADH oxidase	0·27 (79·4)[b]	0·03 (8·8)	0·01 (2·9)	0·03 (8·8)
Succinate dehydrogenase	0·47 (85·4)	0·07 (12·7)	0	0·01 (1·8)
Fumarate reducatase	1·17 (75·5)	0·21 (13·5)	0·05 (3·2)	0·12 (7·7)
NADPH→NAD^+	0·54 (78·3)	0·07 (10·1)	0·05 (7·2)	0·03 (4·3)
NADH→cytochrome c, Rotenone sensitive[c]	0·61 (85·9)	0·08 (11·3)	0·01 (1·4)	0·01 (1·4)
NADH→cytochrome c, Rotenone insensitive[d]	0·16 (33·3)	0·28 (58·3)	0	0·04 (8·3)
Adenylate kinase	0·89 (19·8)	0·18 (4·0)	0·13 (2·9)	3·30 (73·3)
Citrate synthase	0·02 (10·0)	0·01 (5·0)	0·13 (65·0)	0·04 (20·0)
'Malic' enzyme	0·91 (7·2)	0·44 (3·5)	9·14 (72·0)	2·24 (17·6)
Fumarase	0·06 (10·0)	0	0·45 (75·0)	0·09 (15·0)

[a] Units express total activity in μmol/min of mitochondria and mitochondrial fractions derived from 5·0 g of tissue.
[b] Numbers in parentheses express percent distribution of recovered activity.
[c] NADH→cytochrome c activity sensitive to 100 μM rotenone.
[d] NADH→cytochrome c activity insensitive to 100 μM rotenone.

Table 3. Phosphorus content of mitochondrial membranes and the 30–55 fraction of *Hymenolepis diminuta*

Preparation	μg phosphorus/ mg protein
Mitochondrial membranes	32·7
30–55 fraction*	13·2

* Partially lipid depleted preparation, derived from mitochondrial membranes following precipitation between 30 to 55% ammonium sulfate saturation.

Table 4. Stimulation of transhydrogenase activity by phospholipid addition to the 30–55 fraction of *Hymenolepis diminuta*

Phospholipid	Amount (μmol)*	Activity (nmol/min/mg)†
Phosphatidylcholine	none‡	26·1
	0·05	52·4
	0·25	79·5
Phosphatidylethanolamine	none	27·7
	0·05	29·5
	0·25	29·0
Phosphatidylserine	none	31·1
	0·05	30·1
	0·25	31·4

* Indicates μmol of phospholipid added to the 30–55 fraction, in the presence of 0·1% sodium cholate, and subjected to sonication.
† Activities are expressed per mg protein; 0·12 mg protein was employed for each assay.
‡ Material subjected to equivalent treatment without the addition of phospholipid.

by the transhydrogenase was demonstrable using the 30–55 fraction inasmuch as the addition of phosphatidylcholine to this fraction increases activity significantly. Neither phosphatidylethanolamine nor phoshphatidylserine effectively altered transhydrogenase activity (Table 4) (Fioravanti and Kim, 1983).

Using isolated and everted IM vesicles, i.e. SMP, the cestode transhydrogenase was found to catalyze non energy-linked and energy-linked NADH→$NADP^+$ reactions (Fioravanti *et al*. 1992). As presented in Table 5, rotenone addition to *H. diminuta* SMP results in somewhat of an increase in the rate of the NADPH→NAD^+ reaction, but significantly inhibits the NADH→$NADP^+$ reaction as measured by reduction of the appropriate AcPyNAD(P) acceptor (Fioravanti *et al*. 1992). These findings indicate that rotenone inhibits oxidation of NADH or AcPyADH and that an energization of the SMP, via electron transport- associated NADH oxidation, stimulates the NADH→$NADP^+$ reaction. When, *H. diminuta* SMP were treated with Mg^{++} plus ATP in the presence of rotenone, a significant increase in NADH→$NADP^+$ transhydrogenation also was noted as compared to samples containing rotenone alone (Table 6). This increased activity was not matched by the individual addition of either Mg^{++} or ATP. Furthermore, the Mg^{++} plus ATP-dependent increase in NADH →$NADP^+$ activity was lowered to the level observed with rotenone alone when either ethylenediaminetetraacetate (EDTA) or oligomycin were added to the reaction vessels, in keeping with the involvement of Mg^{++}-dependent ATPase. Thus, an energy-linkage was apparent wherein ATP hydrolysis is energizing the mitochondrial membranes, thereby driving NADPH

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Table 5. Reduced pyridine nucleotide utilization by *Hymenolepis diminuta* submitochondrial particles

Reaction	Addition	Activity (nmol min^{-1} [mg protein]$^{-1}$)
NADPH→NAD^+	None	188·0±9·6 (30)
	Rotenone†	217·0±18·4 (12)
NADH→$NADP^+$	None	35·8±1·9 (49)
	Rotenone	14·8±0·6 (43)

Values are mean±S.E.; the number of observations is presented inside the parentheses, 0·03 mg of protein was used for the assays.
† Rotenone concentration was 100 μM.

formation. Collectively, these data made evident, for the first time, the occurrence of non-energy-linked and energy-linked NADH→$NADP^+$ mitochondrial transhydrogenations in the parasitic helminths (Fioravanti *et al*. 1992).

In other eukaryotes, e.g. mammalian (Danielson and Earnster, 1963; Lee and Earnster, 1989) and insect (Mayer *et al*. 1978; Vandock *et al*. 2008) systems, mitochondrial succinate oxidation energizes the reversible NADPH→NAD^+ transhydrogenase, resulting in an increased NADH→$NADP^+$ (energy-linked) activity. As indicated above, *H. diminuta* mitochondria engage in an inner membrane-associated oxidation of succinate (McKelvey and Fioravanti, 1985; Fioravanti and Reisig, 1990). However, the addition of succinate to *H. diminuta* SMP was without appreciable effect on the NADH→$NADP^+$ activity (Table 6). While the data of Fioravanti *et al*. (1992) reflect an energy-linked transhydrogenation driven by either NADH oxidation or ATP hydrolysis, the lack of succinate-dependent energization supports the view that the *H. diminuta* electron transport system does not engage in appreciable site II or site III activity and thus, appreciable site II or site III phosphorylation.

Certainly, the findings of Fioravanti *et al*. (1992) demonstrate that non-energy- and energy-linked NADH→$NADP^+$ transhydrogenations are catalyzed by *H. diminuta* mitochondria. Employing cestode SMP, Park and Fioravanti (2006) characterized these reactions in greater detail. For these studies, and subsequent considerations, the following designations pertain. The NADH→$NADP^+$ reaction measured in the presence of rotenone, is deemed to be essentially non energy-linked and is so designated. The energy-linked transhydrogenations are denoted as follows: (1) the NADH→$NADP^+$ reaction driven by electron transport-dependent NADH oxidation is refered to as ETD; (2) the reaction driven by ATP hydrolysis is designated ATPD. All assessments of energy-linked NADH→$NADP^+$ reactions were corrected for non-energy-linked activity (Park and Fioravanti, 2006).

Table 6. Energy-linked NADH→$NADP^+$ transhydrogenation catalyzed by *Hymenolepis diminuta* submitochondrial particles

Addition*	Activity† (nmol min^{-1} [mg protein]$^{-1}$)
None	14·8±0·92 (43)
$MgCl_2$, 3·0 mM	13·3±2·2 (6)
ATP, 2·0 mM	22·0±3·4 (13)
$MgCl_2$ plus ATP‡	54·1±6·0 (21)
$MgCl_2$, ATP plus EDTA, 5·0 mM	14·8 [13·6, 15·1] (2)
$MgCl_2$, ATP plus oligomycin, 0·5 μg	13·7±4·3 (5)
Succinate, 3·0 mM	16·1±2·7 (10)

* All assays contained 100 μM rotenone.
† Values are mean±S.E.; the number of observations is presented inside the parentheses; numbers in brackets are observed values; 0·03 mg of protein was used for the assays.
‡ Where indicated $MgCl_2$ and ATP were present at 3·0 mM and 2·0 mM, respectively.

The data of Table 7 indicate that the *H. diminuta* SMP non-energy-linked transhydrogenation is unaffected by N,N′-dicyclohexylcarbodiimide (DCCD), a known inhibitor of proton translocating systems, as well as the protonophores carbonyl cyanide 3-chlorophenylhydrazone (CCCP), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and niclosamide (Heytler, 1979; Fisher and Earle, 1982; Hassinen and Vuokila, 1993). All of these inhibitors markedly depressed the cestode ETD and ATPD reactions (Table 7). Although these data do not differentiate the effects of inhibitors on the transhydrogenase or the NADH oxidase and ATPase systems, they are demonstrative of a role of transmembrane proton translocation in the cestode energy-linked transhydrogenations.

The data of Park and Fioravanti (2006) presented in Table 7 reflect a coupling of ATP hydrolysis with the ATPD transhydrogenation. This consideration is made even more convincing by the data given in Table 8. Measurements of ATP hydrolysis, assessed as phosphorus liberated, versus AcPyADP reduction indicate an ATP/NADPH ratio of 1·39 in *H. diminuta*. Interestingly, addition of the multivalent protein, bovine serum albumin, increased the rate of phosphorus formation and the accumulation of AcPyADPH yielding an ATP/NADPH ratio of 1·08. These data form a basis for further studies. Nevertheless, they indicate a stoichiometric relationship of ATP hydrolysis to NADPH formation of about 1 : 1 (Table 8).

An evaluation of the effects of pH on the non-energy-linked, ETD, and ATPD reactions proved of interest (Fig. 2). Whereas the ETD reaction was unaffected over a pH range of 5·0–8·0, the non-energy-linked and ATPD reactions displayed

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Table 7. NADH→$NADP^+$ transhydrogenation catalyzed by *Hymenolepis diminuta* submitochondrial particles

	Activity (nmol min^{-1} [mg protein]$^{-1}$)		
Addition	Non-energy linked	ETD	ATPD
None	10·8±0·5 (40)	15·2±1·1 (70)	17·0±1·5 (42)
DCCD (300 μM)	12·2±1·6 (8)	2·6±0·7 (11)	1·8±0·8 (10)
CCCP (2 μM)	10·4±1·2 (11)	3·5±1·0 (12)	1·9±0·7 (11)
FCCP (2 μM)	9·6±1·2 (7)	5·2±1·0 (9)	1·4±0·6 (8)
Niclosamide (1 μM)	9·8±1·3 (11)	3·3±0·9 (12)	3·4±1·0 (10)

Values are mean±S.E.; the number of observations is presented inside the parentheses. DCCD, N,N′-dicyclohexylcarbodiimide; CCCP, 3-chlorophenylhydrazone; FCCP, 4-(trifluoromethoxy) phenylhydrazone. 0·03 mg of protein was used for the assays.

Table 8. Relationship of ATP hydrolysis and energy-linked NADH→$NADP^+$ transhydrogenation catalyzed by *Hymenolepis diminuta* submitochondrial particles

Addition	Phosphorus (nmol min^{-1} [mg protein]$^{-1}$)	AcPyADPH (nmol min^{-1} [mg protein]$^{-1}$)	Phosphorus/ AcPyADPH (mean)
None	36·1±6·9 (5)	26·0±2·6 (5)	1·39
BSA	54·2 [48·3–60·0] (2)	50·3 [40·8–59·8] (2)	1·08

Where indicated, BSA (bovine serum albumin) was present at 0·5% wt vol^{-1}. Reactions were measured in the presence of 25 μM rotenone. Values are mean±S.E.; the number of observations is presented inside the parentheses, whereas the numbers inside the square brackets are observed values. 0·15 mg of protein was used for the assays.

significant peaks at pH 5·5 and 6·5, respectively. Presumably, the ATPD peak also suggests a more optimal pH for the cestode ATPase. Although these data may well reflect more optimal pH ranges, it is significant that with acidification, the non-energy-linked reaction simulates ATPD activity. Thus, the intriguing notion that the non energy-linked reaction assumes the characteristics of the ATPD activity, via a pH-dependent change in the transhydrogenase (conformational, protonation (Galante *et al*. 1980)), is presented (Park and Fioravanti, 2006).

The above data imply that a proton gradient, established either by the electron transport-dependent utilization of NADH or ATP hydrolysis by the Mg^{++}-dependent ATPase, drives the *H. diminuta* energy-linked, transhydrogenase-catalyzed reduction of $NADP^+$. The possibility that the *H. diminuta* transhydrogenase can act in transmembrane proton translocation was first evaluated by Mercer *et al*. (1999). They proposed that the helminth transhydrogenase, in catalyzing NAD^+ reduction, could act in the simultaneous movement of protons from the matrix to the intermembrane space compartment as reported for the corresponding mammalian transhydrogenase (Danielson and Ernster, 1963; Rydström *et al*. 1975). To this end, *H. diminuta* SMP were evaluated with respect to transhydrogenase-catalyzed proton translocation.

Presented in Table 9 are the effects of protonophores or DCCD on the catalysis of the NADPH→NAD^+ activity of *H. diminuta* SMP. In the presence of protonophores, a marked increase in AcPyAD reduction is apparent while DCCD significantly inhibits the activity, thereby indicating that with hydride ion transfer between NADPH and NAD^+, the enzyme concomitantly catalyzes transmembrane proton translocation into the SMP intravesicular space. The increased activity noted in the presence of the protonophores indicates that the protonophores relieved an inhibition of the NADPH→NAD^+ system created by the enzyme-dependent intravesicular accumulation of protons (Mercer *et al*. 1999).

To assess further the potential of the NADPH→NAD^+ transhydrogenase to act as a proton pump, fluorometric assessments of the *H. diminuta* SMP NADPH→NAD^+ transhydrogenation were performed using as a probe, 8-anilino-1-napthalenesulfonic acid (ANS). Under the conditions employed, the added ANS would be in its anionic form and, thus, attracted to an intravesicular accumulation of a positive charge. In its attraction to this

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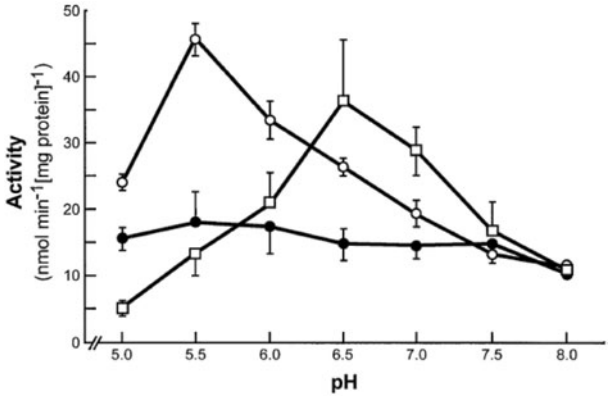

Fig. 2. The effects of pH on the NADH→NADP$^+$ transhydrogenation reactions of adult *Hymenolepis diminuta* submitochondrial particles. Symbols used are: –○–, non-energy-linked; –●–, electron transport–driven; –□–, ATP–driven. Error bars represent S.E. Activities were measured in the absence of BSA. 25 μM rotenone was employed for assessments of the electron transport–(ETD) and ATP–driven (ATPD) reactions. The mean value for the non-energy-linked reaction at pH 8·0 was 11·3 ± S.E. 1·1 whereas that for the ATPD reaction was 10·9 ± 3·2. Values for the ETD reaction at pH 7·5 and 8·0 were 14·8 ± 2·1 and 10·4 ± 1·3, respectively. Numbers of observations were: non-energy-linked – pH 5·0, 8; pH 5·5, 6; pH 6·0–6·5, 5; pH 7·0–8·0, 7. ETD-pH 5·0–6·5, 5; pH 7·0, 7; pH 7·5, 4; pH 8·0, 8. ATPD- pH 5·0, 7; pH 5·5, 8; pH 6·0, 7; pH 6·5, 5; pH 7·0, 7; pH 7·5, 6; pH 8·0, 7. An amount of 0·03 mg protein was employed for assays.

intravesicular environment, ANS fluorescence would be enhanced by its binding to the SMP membrane (Azzi *et al*. 1969). The assay system contained NADPH and NAD, with appropriate substrate generating systems, as well as rotenone and potassium cyanide. As presented in Fig. 3A, with the addition of NAD$^+$ to the assay, a time-dependent increase in ANS fluorescence was noted. In contrast, with additions of the protonophores, i.e. CCCP (Fig. 3B) or niclosamide (Fig. 3C), a rapid collapse in the increasing fluorescence observed in the absence of the inhibitors was apparent. Moreover, with the addition of DCCD to the reaction medium, prior to the start of the reaction with NAD$^+$, increased fluorescence was essentially abolished (Fig. 3D). Accordingly, these data support the concept that the *H. diminuta* transhydrogenase system is a transmembrane component that catalyzes hydride ion transfer with concomitant proton translocation from the mitochondrial matrix to the intermembrane space (Mercer *et al*. 1999).

The findings supporting a concomitant proton translocation with NADPH→NAD$^+$ transhydrogenation in *H. diminuta* mitochondria, in conjunction with those supporting a proton gradient as the driving force for the energy-linked NADH→NADP$^+$ reactions (Fioravanti *et al*. 1992; Park and Fioravanti, 2006), suggested that the helminth

Table 9. Effects of protonophores and DCCD on the NADPH→NAD$^+$ transhydrogenase activity of *Hymenolepis diminuta* submitochondrial particles

Addition	Activity (nmol min^{-1} [mg protein]$^{-1}$)
None	169·6 ± 5·6 (9)
CCCP, 1 μM	237·7 ± 5·7 (6)
FCCP, 1 μM	243·9 ± 5·2 (9)
Niclosamide, 1 μM	214·4 ± 4·2 (8)
DCCD, 100 μM	68·3 ± 7·1 (11)

Values are means ± S.E.; the number of observations is presented inside the parentheses, 0·02 mg of protein was used for the assays. Mean value for each treatment differed significantly when compared to the untreated control.

transhydrogenase, in catalyzing NADH formation, serves as an additional site for anaerobic phosphorylation. In this context, it is noteworthy that bovine heart SMP engage in transhydrogenase-dependent phosphorylation when catalyzing NADPH→NAD$^+$ transhydrogenation in the virtual absence of electron transport activity (Van de Stadt *et al*. 1971; Donstov *et al*. 1972). Mercer-Haines and Fioravanti (2008) evaluated the possibility that the *H. diminuta* transhydrogenase can serve as an additional phosphorylation site using SMP as the enzyme source. Net phosphorylation, measured by ^{32}P incorporation into ATP, was assayed by a modification of the procedure of Saz *et al*. (1972) and employed substrate generating systems in the absence or presence of rotenone and the additives indicated in Tables 10 and 11.

Given in Table 10, are the data obtained wherein reduced pyridine nucleotide-dependent net phosphorylation by *H. diminuta* SMP was assessed. For these studies, exogenous NAD(P)H-generating systems were employed as indicated. Obviously, NADH-dependent phosphorylation was enhanced when fumarate was added to the system and in both the absence and presence of fumarate, rotenone significantly inhibited net ATP generation (Table 10). Significantly, when NADPH was substituted for NADH in measurements of net phosphorylation, either in the absence or presence of fumarate, no phosphorylation was measurable. However, when NADPH served as the source of reducing power, in the presence of NAD$^+$, marked net ATP generation was noted and the degree of this net phosphorylation increased with fumarate supplementation. Collectively, the data of Table 10 support prior findings and demonstrate, based on net ^{32}P incorporation, that: (1) the *H. diminuta* electron transport system indeed displays a preference for NADH; and (2) via the transhydrogenase system NADPH can serve as a source of reducing equivalents for electron transport (Mercer-Haines and Fioravanti, 2008).

Indeed, because the *H. diminuta* NADH→NADP$^+$ reaction is enhanced significantly by

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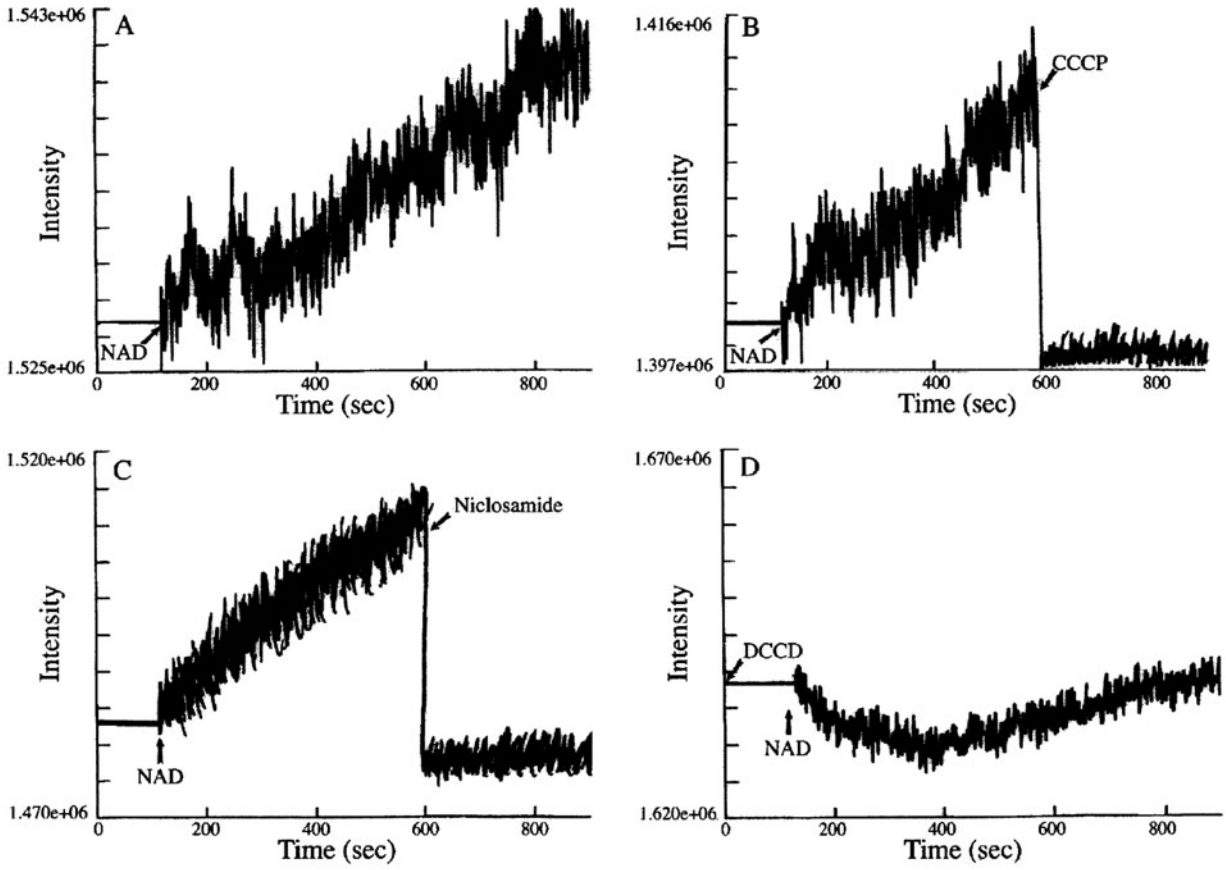

Fig. 3. Enhancement of ANS fluorescence by *Hymenolepis diminuta* SMP-catalyzed NADPH→NAD^+ transhydrogenation. For assays 2·0–3·0 mg protein was employed. (A) Fluorescence enhancement following the start of transhydrogenation with NAD. (B) Quenching of transhydrogenase-dependent fluorescence by 1 mM CCCP. (C) Quenching of fluorescence by 3 mM niclosamide. (D) Inhibition of transhydrogenase-dependent enhanced fluorescence by the addition of 600 mM DCCD prior to the start of the reaction with NAD.

mitochondrial ATP hydrolysis and the NADPH→NAD^+ reaction results in a concomitant movement of protons from the mitochondrial matrix to the intermembrane space, evaluations were undertaken to determine if the cestode NADPH→NAD^+ transhydrogenase, in catalyzing NAD^+ reduction, could serve as an additional site for net ATP generation (Mercer-Haines and Fioravanti, 2008). Employing *H. diminuta* SMP and both NADPH and NAD^+ generating systems as indicated, assessments of the transhydrogenase system serving in the potential generation of ATP were made and these data are given in Table 11. As noted, in the presence of rotenone the catalysis of the NADPH→NAD^+ reaction by the cestode transhydrogenase resulted in an appreciable net ATP generation. Conversely, when these assessments were made in the presence of rotenone with either DCCD or niclosamide supplementation, no ATP generation was detected in keeping with a transhydrogenase-dependent formation of a proton gradient by transmembrane pumping of protons. Within this context, it also is of note that when ^{32}P incorporation was assessed, in the presence of an NADPH generating system, rotenone and NAD^+, in the absence of NAD^+ generation, incorporation was markedly less than that observed in corresponding samples in which rotenone was absent (Table 10) or in which both the NADH generating system and rotenone were present (Table 11). Thus, the need for an ongoing generation of NADPH and NAD^+ when the transhydrogenase serves as a phosphorylation site is apparent. Moreover, if these assessments were made in the presence of rotenone, but without an exogenous NAD^+ generating system, only a low level of phosphorylation (~14% of that noted in the presence of the NAD^+ generating system) was observed. The latter would likely reflect the 'leakiness' of the rotenone inhibitor (Table 11).

Based on the data obtained relating to the *H. diminuta* mitochondrial IM-associated and reversible NADPH→NAD^+ transhydrogenase, the model for the involvement of this enzyme in the energetics of the cestode is presented in Fig. 4. With the oxidative decarboxylation of malate by the 'malic' enzyme, reducing equivalents for electron transport are accumulated in the matrix as NADPH. With NADPH, in the presence of NAD^+, the IM-associated transhydrogenase catalyzes the formation of NADH. This scalar reaction, occurring on the matrix surface

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Table 10. Reduced pyridine nucleotide-dependent phosphorylation by *Hymenolepis diminuta* submitochondrial particles

Addition(s)	^{32}P incorporation nmol/mg protein
NAD[a], 0·24 mM	48·6 ± 2·3 (7)
NAD plus rotenone, 10 μM	6·9 ± 0·42 (7)[b]
NAD plus fumarate, 0·6 mM	90·2 ± 2·6 (6)[c]
NAD, fumarate plus rotenone	5·4 ± 0·93 (4)[b]
NADP[d], 0·24 mM	ND
NADP plus fumarate	ND
NADP plus NAD[e], 0·6 mM	30·6 ± 3·8 (9)
NADP, NAD plus fumarate	55·1 ± 2·8 (9)[c]

Values are mean incorporations after 5 min ± S.E. Numbers of observations are in parentheses. 0·4–0·5 mg protein was employed for assays. ND indicates not detected.
[a] Exogenous NADH-generating system consisted of glutamate (3·0 mM) and glutamate dehydrogenase (5 U).
[b] Significantly different when compared to non-rotenone containing counterparts.
[c] Significantly different when compared to counterparts without fumarate.
[d] Exogenous NADPH-generating system consisted of glucose-6-phosphate (3·0 mM) and glucose-6-phosphate dehydrogenase (5 U).
[e] No exogenous NAD-generating system.

Table 11. NADPH→NAD$^+$ transhydrogenase as an energy-coupling site in *Hymenolepis diminuta* submitochondrial particles

Addition(s)	^{32}P incorporation nmol/mg protein
NADP[a], 0·24 mM, NAD[a], 0·6 mM plus rotenone, 10 μM	35·2 ± 3·1 (17)
NADP, NAD, rotenone plus DCCD[b], 240 μM	ND
NADP, NAD, rotenone, niclosamide, 1·6 μM	ND
NADP, NAD[c] plus rotenone	5·1 ± 0·79 (7)[d]

Values are mean incorporations after 5 min ± S.E. Numbers of observations are in parentheses. 0·4–0·5 mg protein was employed for assays. ND indicates not detected.
[a] Exogenous-generating systems employed were as follows: NADPH, glucose-6-phosphate (3·0 mM) and glucose-6-phosphate dehydrogenase (5 U); NAD, pyruvate (15 mM) and lactate dehydrogenase (5 U).
[b] DCCD, N,N′-dicyclohexylcarbodiimide.
[c] No exogenous NAD-generating system.
[d] Significantly different when compared to counterparts containing an NAD-generating system.

of the transhydrogenase, fosters a concomitant translocation of protons from the matrix to the intermembrane space compartment via the proton-pumping ability of the transhydrogenase. The proton gradient so established serves in driving ATP synthesis by the ATP synthase system. Likewise, the oxidation of transhydrogenase-formed NADH by the cestode electron transport system results not only in fumarate reduction but the establishment of a proton gradient, promoting net phosphorylation. With active malate oxidation, NADPH is formed allowing for NADH accumulation. Coupled to this is the oxidation of NADH via the electron transport-dependent fumarate reductase. With respect to the energy-linked NADPH-forming reactions (i.e. NADH→NADP$^+$), a reversal of the role of the transhydrogenase occurs. The hydrolysis of ATP or NADH oxidation establishes a proton gradient, that with the transhydrogenase catalyzed transmembrane movement of protons, drives NADPH formation (Fig. 4). Therefore, in *H. diminuta* and presumably other helminths, the mitochondrial NADPH→NAD$^+$ transhydrogenase can serve not only as a metabolic connector, but also as a site for anaerobic phosphorylation.

NADH→NAD$^+$ MITOCHONDRIAL TRANSHYDROGENATION IN *HYMENOPEPIS DIMINUTA*

Fioravanti and Saz (1976) first demonstrated the occurrence of a transhydrogenation reaction between NADH and NAD$^+$ (AcPyAD) in *H. diminuta* mitochondria that is independent of the reversible NADPH→NAD$^+$ transhydrogenase. Initial evaluations of this NADH→NAD$^+$ transhydrogenation indicated a predominant IM localization. Furthermore, in adult *Ascaris suum* mitochondria, that apparently lack an NADPH→NAD$^+$ transhydrogenase, an IM-associated NADH→NAD$^+$ transhydrogenation was noted (Fioravanti and Saz, 1976). Two lines of evidence concerning the ascarid NADH→NAD$^+$ transhydrogenation were presented. Firstly, the findings of Rew and Saz (1974) coupled with those of Köhler and Saz (1976) indicated that the nematode, NAD$^+$-preferring 'malic' enzyme was essentially localized in the mitochondrial intermembrane space and that the NADH→NAD$^+$ transhydrogenation could serve in the transmembrane movement of reducing equivalents from the intermembrane space to the matrix compartment. Secondly, the work of Komuniecki and Saz (1979) indicated that the ascarid NADH→NAD$^+$ transhydrogenation predominantly reflected an activity associated with the nematode lipoamide dehydrogenase. Considered collectively, the above noted studies fostered a further evaluation of the mitochondrial NADH→NAD$^+$ transhydrogenation of adult *H. diminuta*.

Employing sonically disrupted *H. diminuta* mitochondria, a comparison was made of the intramitochondrial localizations of the NADH→NAD$^+$ reaction, the NADPH→NAD$^+$ transhydrogenase, the NADH dehydrogenase, and the lipoamide dehydrogenase activities (Walker and Fioravanti, 1995). As expected, the data of Table 12 reveal that

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Table 12. Intramitochondrial localization of NADPH→NAD^+ transhydrogenase, NADH dehydrogenase, lipoamide dehydrogenase, and the NADH→NAD^+ transhydrogenation in *Hymenolepis diminuta*

Reaction	Total Units*			% Recovered Activity	
	Disrupted mitochondria	Membrane fraction	Soluble fraction	Membrane fraction	Soluble fraction
NADPH→NAD^+	2·02 (0·07)†	1·45 (0·11)	0·07 (0·01)	95	5
NADH dehydrogenase	31·80 (1·13)	18·20 (1·37)	1·20 (0·21)	94	6
Lipoamide dehydrogenase	27·97 (0·99)	7·45 (0·56)	11·99 (2·05)	38	62
NADH→NAD^+	10·00 (0·35)	4·78 (0·36)	2·42 (0·41)	66	34

* Units express total activity in μmol/min of fractions from a 3·5 ml preparation of mitochondria equivalent to 28·3 mg protein. Each value is the mean of duplicate assays. Duplicates for each value did not vary by more than 8·5%.
† Numbers in parentheses express activity in μmol min^{-1} $(mg\ protein)^{-1}$; 6·0 μg to 0·12 mg mitochondrial, 8·0 μg to 0·1 mg membrane, and 12·0 to 37·0 μg soluble protein were employed for assay.

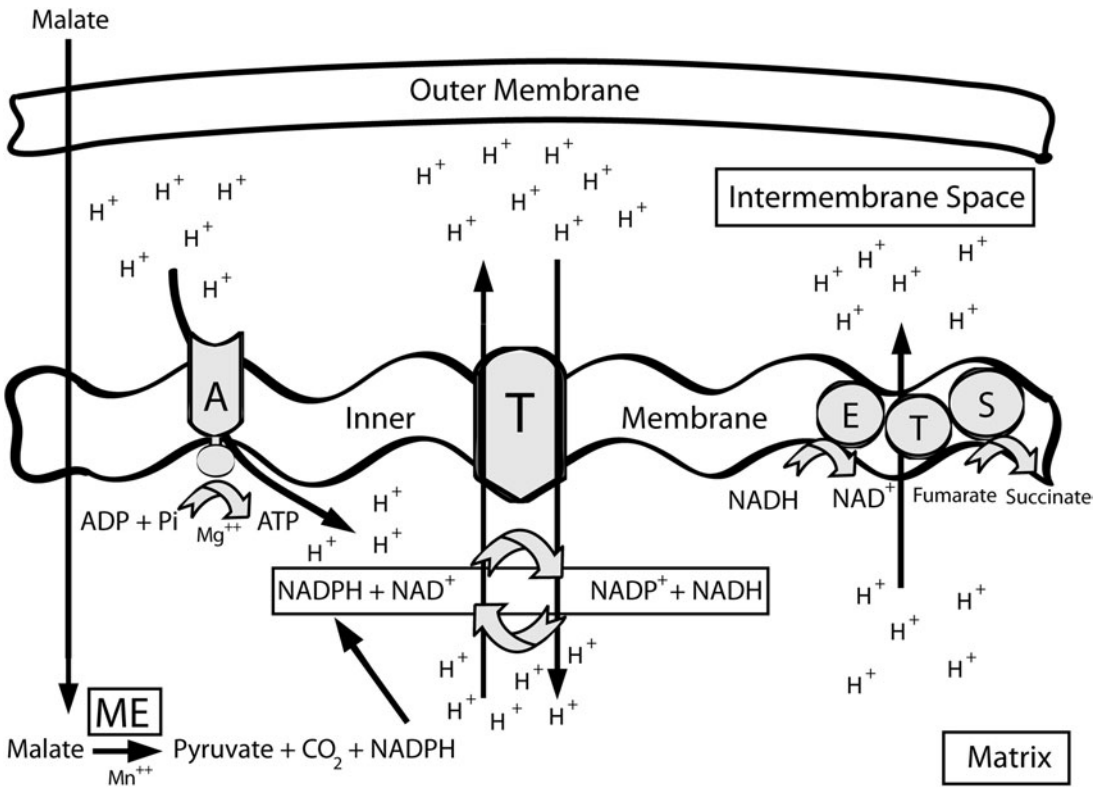

Fig. 4. Model for the role of *Hymenolepis diminuta* inner mitochondrial membrane-associated transhydrogenase. Designations are as follows: T, transhydrogenase; A, ATP synthase/ATPase; ETS, electron transport system; ME, 'malic' enzyme.

both the NADPH→NAD^+ transhydrogenase and NADH dehydrogenase systems are primarily membrane associated. With respect to lipoamide dehydrogenase and the NADH→NAD^+ transhydrogenation, however, the former was predominantly recovered in the mitochondrial soluble fraction (62%) whereas the latter was predominantly membrane-associated (66%). Given the data of Table 12, a further comparison of the four mitochondrial activities was made based on thermal lability.

Isolated *H. diminuta* mitochondrial membranes were exposed to increasing temperatures for five minute periods and these data are given in Fig. 5. NADPH→NAD^+ activity increased up to 35 °C and at 45 °C, activity decreased with complete inactivation at 55 °C. NADH dehydrogenase activity gradually declined at lower temperatures but dramatically decreased at 45 °C with total inactivation at 65 °C (Fig. 5). Whereas lipoamide dehydrogenase activity was stimulated at 25 °C, the activity declined thereafter to about the control level at 45 °C and above 45 °C an increased activity was noted that peaked at 75 °C. Temperatures in excess of 75 °C diminished activity with total inactivation at

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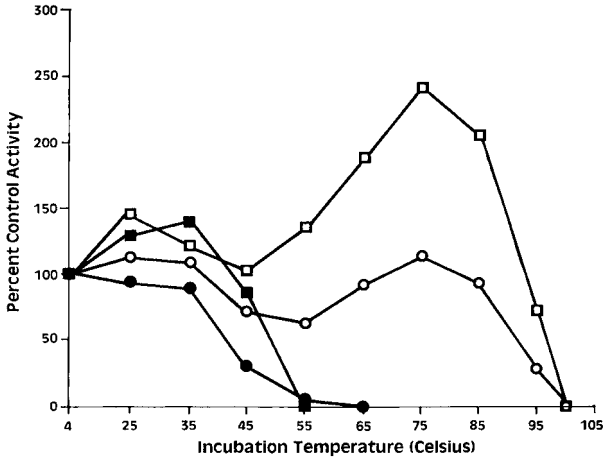

Fig. 5. Thermal profiles of NADPH→NAD$^+$ transhydrogenase, NADH dehydrogenase, lipoamide dehydrogenase, and the NADH→NAD$^+$ transhydrogenation reaction catalyzed by *Hymenolepis diminuta* mitochondrial membranes. Symbols used are: –□–, Lipoamide dehydrogenase; –■–, NADPH→NAD$^+$; –○–, NADH→NAD$^+$; –●–, NADH dehydrogenase.

100 °C. NADH→NAD$^+$ activity appeared intermediate to that of both dehydrogenases. While lower temperatures increased activity, inactivation was apparent at 45 °C and 55 °C. With increasing temperature an increase in NADH→NAD$^+$ activity was noted that exceeded that of untreated membranes at 75 °C and thereafter a diminishment in activity was evident with complete inactivation at 100 °C (Fig. 5). Based on these thermal profiles, in conjunction with the data of Table 12, it appears that the *H. diminuta* mitochondrial NADH→NAD$^+$ transhydrogenation reaction is catalyzed by lipoamide dehydrogenase and possibly by NADH dehydrogenase rather than by an independent transhydrogenase system.

Further studies of the *H. diminuta* NADH→NAD$^+$ transhydrogenation, associated with lipoamide dehydrogenase, were pursued (Walker *et al.* 1997). The occurrence of NADH→NAD$^+$ and lipoamide dehydrogenase activities were assessed for *H. diminuta* cysticercoids, the mitochondria of 6-, 10-, and 14-day-old cestodes, and the mitochondria of pre-gravid/gravid regions of adults (Table 13). All of these preparations, catalyzed an NADH→NAD$^+$ transhydrogenation and lipoamide reduction, with the latter being more prominent. A developmentally related increase in NADH→NAD$^+$ activity is suggested when comparisons of mitochondria from 6-, 10-, and 14-day cestodes are made. In contrast, lipoamide dehydrogenase activity is essentially unchanged, thereby indicating that a system(s) other than lipoamide dehydrogenase contribute to the increased NADH→NAD$^+$ activity as suggested for adult mitochondria (Walker and Fioravanti, 1995). Assessments of NADH→NAD$^+$ and lipoamide dehydrogenase activities for adult *H. diminuta* segments

Table 13. NADH→NAD$^+$ Transhydrogenation and lipoamide dehydrogenase activities of *Hymenolepis diminuta*

	NADH→NAD$^+$ Transhydrogenation	Lipoamide Dehydrogenase
Preparation	Activity: (μmol min^{-1} [mg protein]$^{-1}$)	
Cysticercoids	0·02 ± 0·002 (4)	0·07 ± 0·005 (5)
Helminths		
6-day	0·18 ± 0·006 (8)	0·82 ± 0·035 (8)
10-day	0·27 ± 0·018 (8)	0·88 ± 0·030 (8)
14-day	0·26 ± 0·028 (6)	0·80 ± 0·014 (6)
Helminth regions		
Immature	0·40 ± 0·021 (8)	0·99 ± 0·021 (8)
Mature	0·37 ± 0·025 (8)	0·84 ± 0·019 (8)
Pregravid/ gravid	0·28 ± 0·022 (8)	0·75 ± 0·041 (8)

Values are mean ± S.E.; the number of observations is presented inside the parentheses. 2·5–170 μg protein was used for assessments. For cysticercoids, the supernatant fraction obtained after disruption served as the source of activities, whereas isolated and disrupted mitochondria were used for all other assessments.

indicate that the immature and mature segments have the highest levels of transhydrogenation while the highest level of lipoamide dehydrogenase is with the immature segment (Table 13). The levels of both mitochondrial activities in the immature segment are consistent with it being the most metabolically active region of the adult cestode (Roberts, 1961).

Isolation of the *H. diminuta* mitochondrial lipoamide dehydrogenase was undertaken and a representative purification is presented in Table 14. Utilizing the *H. diminuta* soluble mitochondrial fraction as starting material, coupled with heat treatment and column chromatographies, ~149 fold purification of the enzyme was obtained. As given in Fig. 6, the purified enzyme has a monomeric, M_r of 47 kDa. Via Sephadex G-200 chromatography, the native M_r of the enzyme was estimated to be 93 kDa, consistent with a homodimeric enzyme, and the enzyme's absorption spectra revealed the occurrence of flavin (Walker *et al.* 1997).

A listing of the reactions catalyzed by purified *H. diminuta* lipoamide dehydrogenase and the pH optimum of each of these activities is presented in Table 15. Aside from lipoamide dehydrogenase and NADH→NAD$^+$ transhydrogenation activities, both NADH-dependent ferricyanide reduction (pH 6·5) and a low level of diaphorase (pH 6·0) were detected. Interestingly, the purified enzyme preparation also catalyzed a low level of NADPH→NAD$^+$ transhydrogenation at acidic pH (4·5). An initial comparison of a partial amino acid sequence of the *H. diminuta* lipoamide dehydrogenase indicated that the cestode

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Table 14. Purification of the *Hymenolepis diminuta* lipoamide dehydrogenase

Fraction	Total activity (μmol/min)	Protein (mg)	Specific activity μmol min^{-1} (mg protein)$^{-1}$	Purification	Yield (%)
Mitochondrial supernatant	230·3	122·1	1·9	1·0	100·0
Heat-treated	231·5	26·6	8·7	4·6	>100·0
Concentrated	224·1	17·7	12·7	6·7	97·3
DEAE-Sepharose	133·1	0·51	262·5	138·9	57·8
Sephadex G-100	73·4	0·28	260·4	137·8	31·9
Hydroxylapatite	29·8	0·11	280·9	148·6	12·9

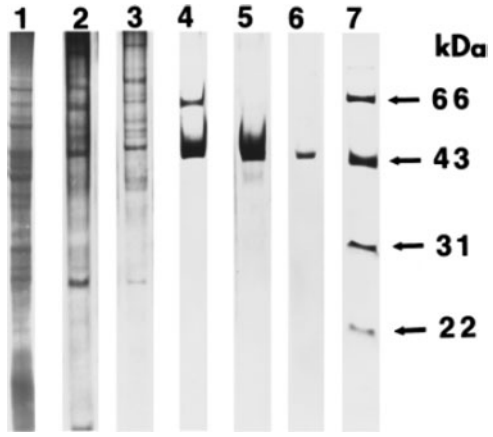

Fig. 6. SDS–PAGE of the purification of *Hymenolepis diminuta* lipoamide dehydrogenase. Lane 1, mitochondrial supernatant; Lane 2, heat-treated; Lane 3, concentrated; Lane 4, DEAE-Sepharose; Lane 5, Sephadex G-100; Lane 6, hydroxylapatite; Lane 7, molecular mass markers. Each lane contained 5 mg protein.

Table 15. Reactions catalyzed by purified *Hymenolepis diminuta* lipoamide dehydrogenase

Reaction	Activity: (μmol min^{-1} [mg protein]$^{-1}$)	pH optimum
NADH→NAD$^+$	54·6	7·5
Lipoamide dehydrogenase	280·9	6·5
NADPH→NAD$^+$	3·8	4·5
NADH diaphorase	2·1	6·0
NADH→ferricyanide	28·1	6·5

Buffers employed were as follows: lipoamide dehydrogenase, NADH diaphorase and NADH→ferricyanide, 100 mM potassium phosphate; NADH→NAD$^+$, 100 mM Tris-HCl; and NADPH→NAD$^+$, 100 mM acetate.

enzyme was most similar to the corresponding enzymes of other parasitic helminths.

Employing purified *H. diminuta* lipoamide dehydrogenase as the antigen, polyclonal rabbit antibodies, directed against this enzyme, were prepared in conjunction with David Upite (unpublished observations). IgG was purified from the rabbit anti-lipoamide dehydrogenase serum and was employed to assess antigen retention in electron micrographs of thin sections of mouse liver mitochondria using the 'HACH' polymer as the embedding material (Olesen *et al.* 1997). The effective use of the IgG prepared using *H. diminuta* purified lipoamide dehydrogenase made evident an appreciable degree of similarity of the *H. diminuta* enzyme with the corresponding mammalian mitochondrial system.

CONCLUSIONS

The mitochondrial IM-associated reversible NADPH→NAD$^+$ transhydrogenase appears to be a crucial component in the energetics of adult *H. diminuta*. Certainly, the service of this transhydrogenase system as a metabolic connector coupling NADP$^+$-linked 'malic' enzyme with the NADH-preferring anaerobic electron transport system is apparent. Furthermore, the more recent findings indicating that the NADPH→NAD$^+$ transhydrogenase, in its action as a proton translocating entity, serves in generating a proton gradient sufficient to support net ATP synthesis is clearly of interest. For the first time in any of the helminths, this enzyme is implicated as an additional site for anaerobic mitochondrial phosphorylation. The interaction of the transhydrogenase system with an established proton gradient also is made evident in terms of the cestode energy-linked transhydrogenations resulting in NADPH accumulation. Given these findings, it would be presumed that specific chemotherapeutic disruption of the helminth mitochondrial transhydrogenase would act to destroy the parasite.

The occurrence of a mitochondrial NADPH→NAD$^+$ transhydrogenase and NADH→NAD$^+$ transhydrogenation has been reported for other cestodes (*viz. Spirometra mansonoides*, Fioravanti and Saz, 1978; *Hymenolepis microstoma*, Fioravanti, 1982*b*; *Taenia crassiceps*, Zenka and Propkopic, 1988) and the nematode, *Setaria digitata* (Unnikrishnan and Raj, 1995). NADPH→NAD$^+$ transhydrogenase activity also has been noted for the trematodes

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Fasciola gigantica, (Umezurike and Anya, 1980) and *Fasciola hepatica* (Watson and Fioravanti, unpublished observations). It will be of interest, therefore, to determine the impact of the NADPH→NAD^+ transhydrogenase on the energetics of these other systems. In light of the potential that an NADH→NAD^+ activity could act as an IM shuttle for reducing power, further evaluations of this reaction in the parasitic helminths are merited.

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