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The Role of the Suprapharyngeal Ganglia in Regulation of Oxidative Metabolism in the Intestinal Mitochondria of Lumbricus Terrestris

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THE ROLE OF THE SUPRAPHARYNGEAL GANGLIA IN REGULATION OF OXIDATIVE METABOLISM IN THE INTESTINAL MITOCHONDRIA OF LUMBRICUS TERRESTRIS

by

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INTRODUCTION

Neuroendocrine cells present in the suprapharyngeal ganglia of Lumbricus terrestris were originally described by Sharrer and Sharrer (1937). Although there have been various neurosecretory cells described in several species of Oligochaetae it was concluded that these differences were a function of age and secretory activity and that basically there were two types. (Meerlant-Meewis, 1959). On the basis of size, location and appearnce, the neurosecretory cells in Lumbricidae have been designated a-cells and b-cells (Goudie, 1968; Marapao, 1959).

Neurosecretory substances have been shown to play a role in regulation of several metabolic processes in Oligochaetae (Golding, 1974). Such as Goudie (1968) demonstrated that neurosecretory substances played a role in osmoregulation in L. terrestris because osmotic stress resulted in a loss of granules from the a-cells. Evidence of a hyperglycemic factor from the suprapharyngeal ganglia of L. terrestris has been presented (Lawrence, Clough, and Craig, 1972). Brain removal resulted in a drop of blood glucose levels to zero and injection of worms with whole brain homogenate resulted in a significant elevation of blood sugar compared to control worms. Vandenbosch (1970) using column and thin layer chromatography characterized this active factor as a ninhydrin positive substance which was probably a polypeptide.

It has been demonstrated that there was a significant increase

in oxygen consumption of intestine from three day debrained worms when compared to control worms. This same effect has also been demostrated for tissue from body wall (Nelson, 1969; Papoe, 1973). In addition, Papoe (1973) showed that incubation of these tissues from debrained worms with either crude brain homogenates or partially purified brain extract from which momoanimes had been removed cause a dose related increase in oxygen consumption.

Additional evidence for the role of invertebrates neuroen docrines in the regulation of oxidative metabolism has been obtained in insects. Keeley and Friedman (1967; 1969) and Keeley (1970) have shown a depression of oxygen consumption of fat bodies from 30 day cartiacectomized male cockroaches. It was further demonstrated that fat body mitochondria from cardiaectomized animals also showed a lower oxygen quotient and decreased levels of electron tansport enzymes that mitochondria from control animals.

The factors, presumable neurosecretory in origin, affecting oxidative metabolism in earthworms and insects may have an analagous function to the mammalian hormone thyroxine. Studies have shown the respiratory function of mammalian hormone thyroxine. Studies have shown the respiratory function of mammalian liver mitochondria to be decreased by thyroidectomy and to be increased in hyperthyroid rats (Pittot and Yatvin, 1973).

In invertebrates the insect fat body has been shown to carry on many of the functions of mammalian liver and to be an analagous organ (Kilby, 1965). In the earthworm L. terrestris use in the present

study the anterior intestine containing chloragocytes have been found also to be analogous in function to the mammalian liver in the ability to store glycogen, lipids and possible function in nitrogen metabolism (Roots, 1959). It is for these reasons that the anterior portion of intestine was chosen for use in these experiments.

The purpose of the present investigation was to determine if the lowering of oxygen consumption in intestinal tissue from three day debrained worms was the result of some changes in the mitochondria or due to alteration and/or damage at some other cellular site not involving the mitochondria.

To answer this question the oxygen quotient of whole intestinal tissue and intestinal mitochondria from debrained and control worms were monitored in basic media and following the addition of key intermediates.

REVIEW OF LITERATURE

Until recently there has been little knowledge of regulation of oxidative metabolism in invertebrates. Elizinga (1963) compared the whole body oxygen consumption of the earthworm Lumbricus terrestris from zero to seventeen days post debraining with oxygen consumption of control worms. Initially the oxygen consumption of the debrained worms decreased compared to the control worms. The depression reached a maximum at three days after debraining. Following this, oxygen consumption increased but did not return to normal levels.

Nelson (1969) measured the oxygen consumption of body wall and intestinal tissue from normal and debrained worms. He found the oxygen quotients of intestine to be twice the level of oxygen quotients from the body wall. After debraining the oxygen quotients of intestine decreased for the first three days and was significantly different from controls three days after debraining. Tissue from body wall responded in a similar manner. Nelson (1969) also found that addition of glucose (.33mM) to the assay media containing tissue samples from intestine and body wall from three day debrained worms caused no significant increase in oxygen quotient. This indicated that depression of intestianal and body wall tissue from three day debrained worms was not due to lack of glucose in body fluids.

When whole brain homogenate in a concentration of one brain per mililiter was added to the bathing media a significant increase in the oxygen quotient of intestine from both three day and control worms

was observed (Papoe, 1973). When this amount of brain homogenate was doubled a dose response was observed with the intestinal tissue. In addition Papoe (1973) showed that incubation of tissue with a water extract of brain tissue from which monoamines had been removed resulted in a general increase in the oxygen quotient (QO_2) of intestinal tissue. Rat brain extracted by the same procedure did not have this effect on the oxygen quotient of the tissue. The effect of the moncamines serotonin, dopamine, adrenalin and noradrenalin on the QO_2 of body wall and intestine were also determined. Serotonin and dopamine had the greatest effect on the QO_2 of both tissues used. It was proposed that either of these two amines or both may play a hormonal role in metabolic regulation of the earthworm L. terrestric. Papoe (1973) also noted that intestinal and body wall tissue from worms decapitated by removal of the anterior segments prior to dissection had a oxygen quotient lower than tissue from worms that were not decapitated before disection. The QO_2 of decapitated controls was 22.0% lower for body wall compared to 25.0% lower for intestine. No significant drop was noted in tissue from decapitated three day debrained worms. This would indicate that the trauma of disection was apparently being mediated by sensory input through the central nervous system causing a release of a substance that elevated the oxygen consumption of the body wall and intestine.

Kale and Rao (1973) have shown a role for neurosecretory substances in temperature acclimation in some species of tropical oligochaetes. Injection of CNS homogenate from cold acclimated worms into

warm adapted worms caused a elevation whole body oxygen consumption in the warm aclimated worms.

Further evidence for neuroendocrine regulation of oxidative metabolism in invertebrates has been obtained in arthropods. Silverthorn (1973) showed that removal of eyestalks or sinus glands from the fiddler crab (Uca pugalata) eliminated seasonal differences in respiration caused by premeasurement thermal history. Extracts of eyestalk gland from cold acclimated animals caused an increase in whole body oxygen consumption when injected into warm adapted animals (Silverthorn, 1975a). This same effect was also noted for tissue for gill and hepatopancreas (Silverthorn, 1975b).

More information is available about the control of oxidative metabolism in insects. Keeley and Friedman (1967) have shown a lowered whole body of oxygen consumption in thirty day cardiaectomized male cockroaches. Studies on dry body weight indicated that thirty day cardiaectomized animals are unchanged as compared to control with respect to percent dry matter and water content. From this it was concluded that the operated animals were trophically normal and the lower oxygen consumption was due to a metabolic effect. They also observe that fat body oxygen consumption was lowered 47% in thirty day cardiaectomized animals. No respiratory differences were seen in muscle from either long term or control animals. Addition of the key intermediates alpha-ketoglutarate and pyruvate did not cause a return of oxygen consumption of fat bodies from thirty day cardiaectomized animals back to normal (Keeley and Friedman, 1969). Keeley

and Waddill (1971) in further studies have shown a significant lowering of oxygen quotients of fat body mitochondria from thirty day cardiaectomized animals. Cellular concentrations of cytochrome c reductase and cytochrome c oxidase were also reduced (Keeley, 1972). Keeley and Waddill (1971) have also shown that daily injection of acts following the thirty days after cardiaectomy raised whole body oxygen consumption of cardiaectomized animals but these extracts have no effect when added to in vitro systems of mitochondria from thirty day cardiaectomized animals. They concluded that the active agent was a neurosecretory substance produced by the n-cells in the CNS and released from the corpra cardiaca. Recently, Keeley (1974) has shown evidence that manipulation of the mouth parts associated with feeding may be a stimulus for a release of neurosecretory substance that would cause an increase in oxidative capacity of the fat body mitochondria.

Although the presence of glycolysis, citric acid cycle and electron transport systems have been documented for many species of arthropods and some species of molluscs, little information is available on the pathways of carbohydrate metabolism in Annelida (Laverack, 1963). Glucose-1-phosphate has been shown to be present in annelids but other glycolytic intermediates have not been reported (deLey and Vercruysse, 1955).

Pyruvic acid and alpha-ketoglutarate have been found in fairly large quantities in two species of annelids (Petrucce, 1955). When pyruvate and alpha-ketoglutarate were added to homogenates of the

earthworm P. velutinus a large increase in oxygen consumption was noted. Dastoli (1964) has shown the presence of enzmes of the citric acid cycle and glycolysis using homogenates of body wall of L. terrestris. These enzymes seemed to be similar in function to enzymes of mammalian systems. Saroja and Rao (1965) have studied the levels of succinic dehydrogenase from annelid body in response to temperature. The activity of this enzyme was elevated in cold-adapted worms.

Although iodine is taken up by the earthworm and other annelids there is no evidence for any thyroxine-like compounds, be in structural proteins (Fletcher, 1969). Neurosecretory substances involved in regulation of oxidative metabolism in insects and earthworms may be analagous to mammalian thyroxine. Liver mitochondria from hyperthyroid rats showed an increased level of cytochrome c and eletron transport activity (Volpin, Kaplay and Sanadi, 1969). Addition of thyroine, in vivo increased synthesis of mitochondrial protein (Kaiser and Bygrove, 1969). Thyroidectomy decreases both the cyotchrome C content and oxygen consumption of rat muscle (Klitgaard, 1966). Liver mitochondria isolated from rats thyroidectomized six weeks previously show a 45 to 50 percent decrease in oxidation rates of Krebs cycle intermediates (Bronk, 1966). This evidence supports this assumption that the mammalian thyroid hormone exerts its influence on the basal metabolism of mammals by an effect on the oxidative capacity of the mitochondria.

MATERIALS AND METHODS

General Procedure

Specimens of the earthworm, Lumbricus terrestris, used for this investigation were obtained from a local bait dealer in Kalamazoo, Michigan. The animals were stored in a commercial bedding and kept at a temperature of 5 to 7°C. Only worms with prominent clitellae, an indication of maturity, were used in this investigation. Tissues from three day debrained, ten day debrained and control worms were used for measurement of mitochondrial oxygen consumption. The following general procedures were used on all worms unless otherwise stated. All experiments were performed between 9:00 Am. and 1:00 P.M.

Preparation of tissue

Worms were removed from the refrigerator, rinsed with water and separated into two groups. One group was debrained and the other was not debrained. The brain was removed by making a mid dorsal incision in the first three to four segments. The ganglia were exposed and the entire bilobed structure was removed. After debraining the worms were wrapped in moist paper towels and returned to the refrigerator for periods of three or ten days. Control worms, with the exception of debraining, were treated in the same manner.

After either three or ten days both groups of worms, experimental and control worms were removed from the refrigerator and the intestine was assayed for oxygen consumption. Before removal of the

intestine all worms were decapitated by removal of the first eight to ten segments. This was done to remove the brain as a source of neurosecretory substance that might be released due to the trauma of dissection (Papoe, 1973). A dorsal incicsion was made from the clitelum to the last posterior segment. The body wall was pinned back. The intestine was cut open and the intestinal contents were washed out with saline. The intestinal segments corresponding to section II (Tillinghast and MacDonnell, 1972) were removed by cutting the intersegmental connections and washed again after excission to remove any remaining contents and/or digestive enzymes. Care was taken that no portion of the ventral nerve cord adhered to the intestine.

Manometric measurement of oxygen consumption

After removal, intestinal samples were placed in the main compartment of a sixteen mililiter manometric reaction flask containing three mililiters of basic media consisting of worm saline (Drewes and Pax, 1973). Containing 20 mM gluclose (Table 1). Glucose was added to ensure that both experimental and control tissue were in the presence of saturation levels of glucose. The center wall contained two tenths mililiter of ten percent potassium hydroxide and fluted filter paper wick.

TABLE 1

Composition of earthworm saline used in the basic media.

Compound	Concentration (mM)
Na^{+}	77.0
Cl^{-}	43.0
K^{+}	4.0
Mg^{++}	1.0
Ca^{++}	6.0
Tris	2.0
$SO_4^{=}$	26.0
Sucrose	55.0

pH was adjusted to 7.4 using concentrated HCL.

In cases where additives were placed in the side arm, the volume of the basic media was reduced an amount equal to that added from the side arm. After preparation, all reaction flasks were kept on ice until used for measurement of oxygen consumption. The time for the preparation of one flask was approximately five minutes.

Oxygen consumption of the intestinal tissue was measured using a Gilson differential respirometer. The gas used was air and a temperature of 15 $\pm$ .5°C was maintained. The flasks were taken from

the ice bath and attached to the manometer arms. A reference flask was attached with a gas volume equal to the total volume of gas in all the reaction flasks. After all flasks were attached, the system was allowed to come to temperature equilibrium for thirty minutes with the operational valves open to the atmosphere. After thirty minutes the operational valves were closed and oxygen consumption was recorder at one hour intevals for a three hour period. In cases where addition of substrates from the side arm was made the substrate was tipped from the side arm into the main compartment at the end of the equilibrium period at the time the operational valves were closed.

After three hours the tissue was removed from the flasks, blotted dry and placed on preweighed foil strips. The tissue was dried on an Ohaus desicator at a seven watt setting for a period of twenty minutes. The drying period was determined from a calibration curve (Figure 1). After drying the tissue was weighed on a Mettler analytical balance.

The results from all manometric experiments were expressed as oxygen quotients denoting the microliters of oxygen consumer per hour per gram of dry tissue weight. The values from oxygen consumption data were corrected for standard temperature and pressure. Mean oxygen quotients for the three hour period were calculated and were used for all analysis.

Isolation of intestinal mitochondria

After removal from the worm, as described earlier, intestinal tissue was placed in ice cold isolation media (20mM sucrose, 5mM Tris,

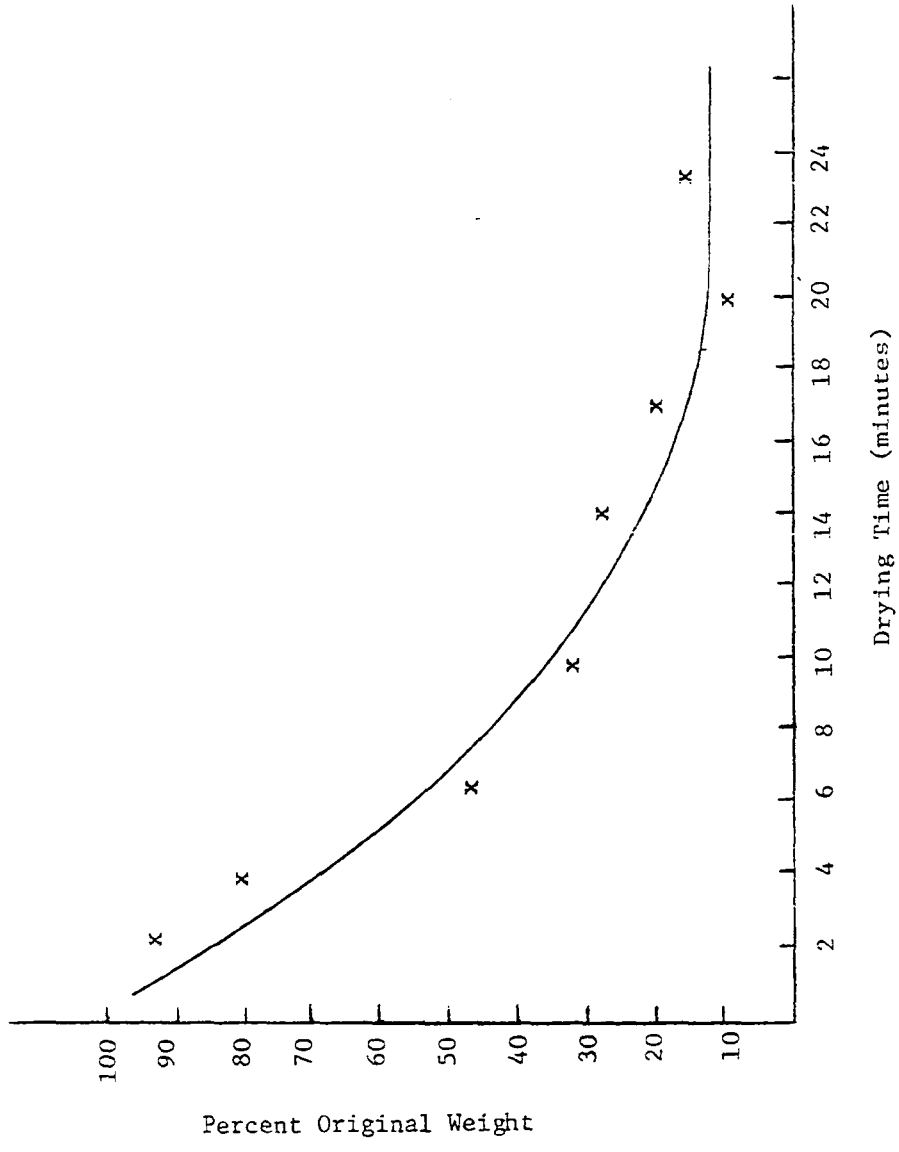


Figure 1: Curve used for determining drying time of earthworm intestinal tissue.

0.8% Bovine serum albumin (BSA), pH 7.4). All solutions were made in double distilled, deionized water. The intestines from twelve to fifteen individuals were pooled, then homogenized with ten mililiters ice cold isolation media per gram of tissue in a hand held glass homogenizer with four to six strokes. Mitochondria were isolated by slight modification of techniques developed by Johnson and Lardy (1967) for rat liver mitochondria. The homogenate was centrifuged at 700 g for ten minutes. All centrifugations were performed at two degrees centigrade in a International refrigerated centrifuge. After the first centrifugation the supernatant was carefully decanted and centrifuged a 8,500 g for fifteen minutes. The supernatant was decanted and discarded. The mitochondrial pellet was suspended in 0.25 M sucrose by passage through the bore of a 0.1 ml pipette. This suspension was centrifuged at 8,500 for fifteen minutes. This step was repeated after which the supernatant was decanted and discarded. The mitochondrial pellet was resuspended in 0.25 M sucrose by passage through a 25 gauge hypodermic needle. This suspension was kept on ice until used for the assay. The total time between isolation of mitochondria and assay was less than one hour.

Polaragraphic measurement of oxygen consumption

Oxygen consumption of mitochondria was determined using a Clark stationary oxygen electrode coupled to a Gilson oxygraph recorder according to the methods of Eastabrook (1967). One and three tenths mililiters of reaction buffer (17mM $K_2H\ PO_4$, 5mM Tris, 5mM $MgCl_2$;

0.8% BSA; pH 7.4) and 0.1 ml of mitochondrial suspension were added to the reaction vessel and allowed to come to equilibrium for five minutes. One tenth mililiter of substrate either succinate or pyruvate was added to give a final concentration of 20 mM to start the reaction. The reaction was allowed to run for five minutes. The rate of reaction was determined by measuring the pen fall during the five minute period. Polaragraphic calibration was made using air-saturated distilled water. All assays were performed at 15°C. Mitochondrial protein was measured using the method of Lowery (1951). Oxygen quotients were expressed as micromoles of O_2 used per hour per miligram of mitochondrial protein.

Experimental Procedures

Oxygen consumption of mitochondria from intestinal tissue of three day debrained and control worms.

The tissue was dissected, mitochondria isolated and the assay performed as described earlier. Either sodium succinate or pyruvic acid (20mM) were used as substrates.

Effect of whole brain homogenate on the oxygen consumption of intestinal mitochondria from three day debrained and control worms.

In all cases succinate (20mM) was used as a substrate. Brains were homogenized in cold reaction media and the volume was adjusted to give a final concentration in the reaction vessel of two brains per mililiter.

Oxidation of metabolic substrates by earthworm intestinal mitochondria.

The substrates succinate, alpha-ketoglutarate, pyruvate citrate and malate were tested to determine if they would be oxidized by earthworm intestinal mitochondria. All substrates were added in a solution made with basic reaction media. Oxidation rates were determined by measuring oxygen consumption by the polaragraphic method.

The effect of respiratory inhibitors on the oxygen consumption of intestinal mitochondria.

To test the integrety of the mitochondria, the effect of respiratory inhibitors KCN and antimycin on oxygen consumption of earthworm intestinal mitochondrial preparation were studied. The inhibitor KCN is specific for the cytochrome a-3 portion of the respiratory chain. It combines irreversibly with it and prevents reoxidation of any other component of the system thus inhibiting oxygen uptake (Quastrel, 1963). KCN was added to give a final concentration of 10^{-3}. The action of the inhibitor antimycin is in inhibiting the flow of electrons from succinate to cytochrome C but not to other acceptors in the mitochondrial system. The inhibition of succinic acid oxidation in the respiratory chain by antimysoin appears to be localized between cytochrome b and c (Quastreal, 1963). Antimyosin was added in a final concentration of 10^{-6} M. Antimyosin was added in a 75% ethanol solution. The final concentration of ethanol in the reaction media was less than 1%. Because of its high affinity for the reaction vessel, the vessel was rinsed three times with 75%

ethanol solution between experiments when antimyosin was used as an inhibitor. The reaction was allowed to run for two minutes after which the inhibitors were added in a 20uL volume. Inhibition was noted by a significant decline in rate of oxygen consumption as determined by a decrease in rate of pen fall on the polaragraph record.

The effect of key intermediates on oxygen consumption of intestinal tissue of three day debrained and control worms.

Tissue was prepared for manometric determination as described previously and assayed for oxygen consumption using manometric tecniques. Either pyruvate or alpha-ketoglutarate was added in a 0.2 ml volume from the side arm to give a final concentration of 20 mM.

Effect of key intermediates on oxygen consumption of intestinal tissue of ten day debrained and control worms.

Tissue from ten day debrained and control worms was prepared for manometric determination of oxygen consumption as described earlier. Pyruvate or alpha-ketoglutarate were added in a 0.2 ml volume from the side arm to give a final concentration of 20mM.

Polaragraphically determined oxygen consumption of whole intestinal - tissue from control and three day debrained worms using key intermediates.

Polaragraphic determination of oxygen consumption have been shown to have two major advantages over the manometric methods (Eastabrook, 1967). There is a great increase in sensitivity ranging from ten to one hundred times over that of a Warburg type apparatus. Also the

speed of maesurement and continuity of recording are distinct advantages over the gasometric methods. Because of these two advantages the effect of key intermediates on the QO_2 of intestinal tissue from three day debrained and control worms were determined using polaragraphic techniques and compare with the results of manometric methods. Intestinal tissue was prepared as for manometric determination as described above. The basic reaction media used in this experiment consisted of salin (Drewes and Pax, 1973) plus 20mM glucose. The media was allowed to come to temperature equilibrium (15°C) after which 250 mg sample of intestine was added and the oxygen consumption was determined by the amount of pen fall. Tissue were observed to continue this endogenous respiratory activity for periods in excess of twenty minutes. For the determination of the effect of key intermediates, either pyruvate or alpha-ketoglutarate were added to give a final concentration of 20mM. The total volume of the reaction mixture was 1.5 ml. All measurements were made at 15 C.

In one set of experiments using the above procedure, no preincubation of the tissue was performed. In a second set of experiments a two hour preincubation was performed at 15°C in basic media before measurement of oxygen consumption was made. Results of all experiments were expressed as uM O_2 used per hour per gram dry tissue weight.

RESULTS

Several metabolic intermediates were tested to determine if they would be oxidized by the earthworm intestinal mitrochondrial preparation used in these experiments. The results are summarized in Table 2. Succinate (20mM) showed the highest oxidation rate of 1.25. Citrate oxidized at the second highest rate (0.42). In descending order of oxidation rate were pyruvate (0.38), alpha-ketoglutarate (0.36) and malate (0.26).

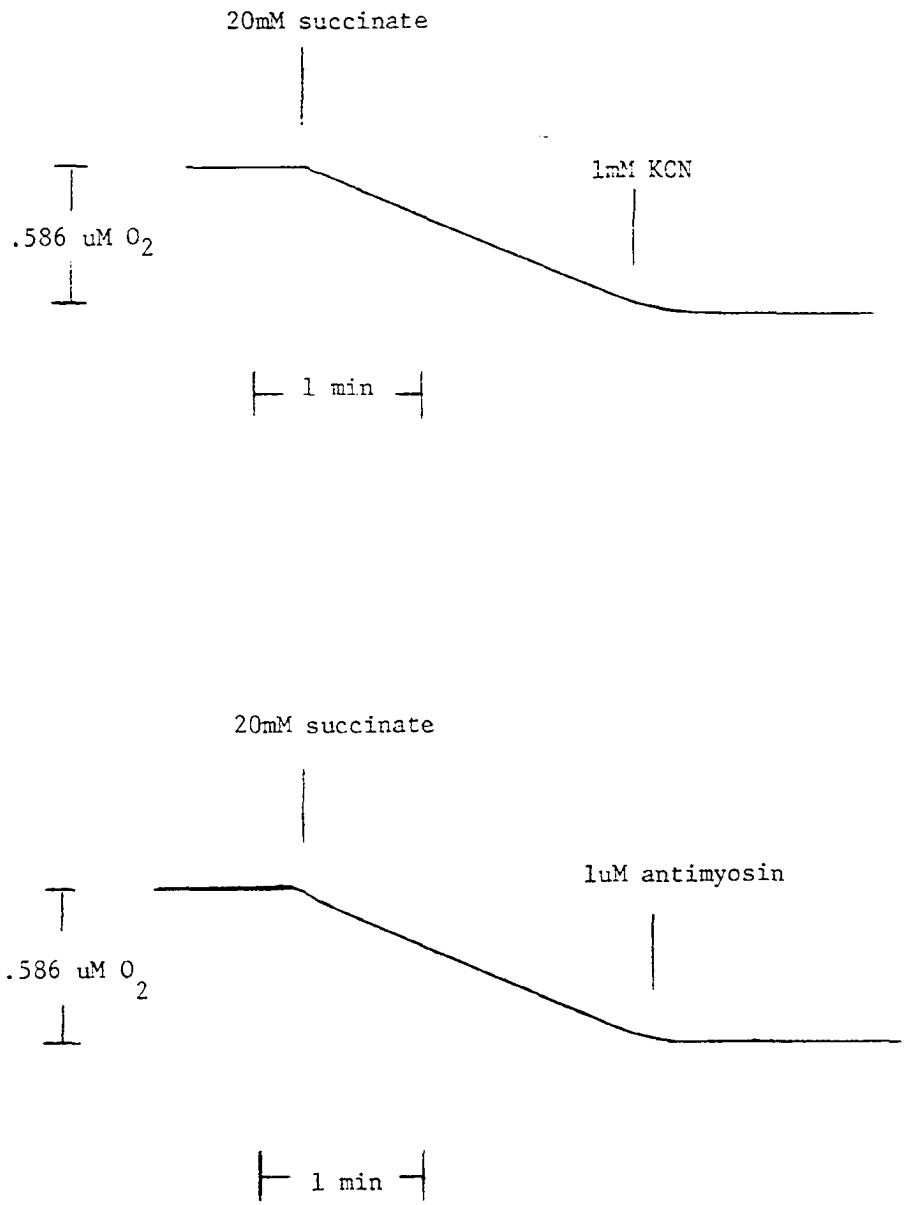
TABLE 2

Oxidation rate of metabolic intermediates by mitochondria from earthworm intestine.

Substrate	QO_2 (uM O_2/hour/mg protein
succinate	1.25
citrate	0.42
pyruvate	0.38
ketoglutarate	0.36
malate	0.26

The effect of the metabolic inhibitors KCN and antimyosin on the oxygen consumption of intestinal mitochondria is shown in Figure 2. The addition of 1mM KCN three minutes after the addition of succinate

Figure 2: Effect of Metabolic Inhibitors on Oxygen Consumption of Earthworm Intestinal Mitochondria



to start the reaction caused a complete inhibition of further oxygen up take as shown by cessation of pen fall on the polaragraph record. Complete inhibition of oxygen consumption is also noted after the addition of 1 uM antimycin. The action of the two respiratory inhibitors on the earthworm mitochondrial preparation indicates that this preparation is a least similar in function to the mammalian systems and that they are intact.

The oxygen quotient of mitochondria from three day debrained and control worms are presented in Table 3. The oxygen quotient of

TABLE 3

Oxygen consumption of mitochondria from three day debrained and control worms.

QU_2 (uM O_2/hour/mg/protein)	
Control	Three day debrained
1. 1.84	1. 2.17
1. 1.85	1. 1.53
2. 1.83	2. 2.38
2. 2.14	2. 2.38
3. 2.18	3. 2.15
3. 2.46	3. 1.79
4. 1.85	4. 1.55
4. 1.85	4. 1.55
5. 2.24	5. 1.13
5. 2.68	5. 1.53
6. 1.74	6. 1.38
6. 1.34	6. 1.38
$X = 1.99 \pm 0.358$	$X = 1.65 \pm 0.642$

mitochondria from control worms had a mean value of 1.99 $\pm$ 0.358. The three day debrained worms had a mean value for mitochondrial oxygen quotient of 1.65 $\pm$ 0.642. Six replicate pools with duplicate determinations were used to test the homogeneity of the sample pools that consisted of mitochondria extracted from several individuals. These determinations showed the pools to be homogenious and in other experiments duplicates were not used. Using one way analysis of variance no significant difference between the two groups was detected ($P > .05$).

Results summarized in Table 4 show the effect of whole brain homogenate on intestinal mitochondria form control worms. Determinations of QO_2 were performed on paired aliquots from six mitochondrial

TABLE 4

Effect of whole brain homogenate on oxygen consumption of intestinal mitochondria from control worms.

QO_2 (uM O_2 hour/mg protein)	
basic media	basic media plus 2 brains per ml
1. 1.50	1. 1.34
2. 1.15	2. 1.59
3. 1.52	3. 1.38
4. 1.03	4. 1.33
5. 1.07	5. 0.76
6. 0.91	6. 0.78
X = 1.19 $\pm$ 0.25	X = 1.19 $\pm$ 0.34

pools. Oxygen quotient of mitochondria assayed in basic media without whole brain homogenate had a mean value of 1.19 $\pm$ 0.25. Mitochondria assayed in basic media plus whole brain hole brain homogenate showed a mean oxygen quotient of 1.19 $\pm$ 0.34. No significant difference between the means was noted ($P > .05$) using a paired t-test.

Results of the effect of whole brain homogenate on oxygen consumption of intestinal mitochondria from three day debrained worms are presented in Table 5. Measurements were done on paired samples from

TABLE 5

The effect of whole brain homogenate on oxygen consumption of mitochondria from three day debrained worms.

QO_2 (uM O_2/hour/mg protein)	
basic media	basic media plus 2 brains per ml
1. 2.13	1. 0.86
2. 0.88	2. 1.36
3. 1.56	3. 1.30
4. 1.49	4. 1.48
5. 1.59	5. 1.11
6. 1.02	6. 1.02
X = 1.44 $\pm$ 0.45	X = 1.19 $\pm$ 0.24

the same mitochondrial pool. The oxygen quotient of mitochondria assayed in basic media without whole brain homogenate had a mean oxygen

quotient of 1.44 ± 0.45. The mitochondria assayed in basic media plus whole brain homogenate had a slightly lower oxygen quotient of 1.19 ± 0.24. No significant difference was detected between the two means using a paired t-test.

Intestinal mitochondria from three day debrained and control worms were shown to oxidize pyruvate (Table 6). The oxygen quotient of

TABLE 6

Oxygen consumption of mitochondria from three day debrained and control worms with pyruvate as a substrate.

QU_2 (uM O_2/hour/mg protein)	
control	three day debrained
.466	.543
.222	.389
.415	.231
.441	.450
.339	.379
X = .376 ± .099	X = .389 ± .114

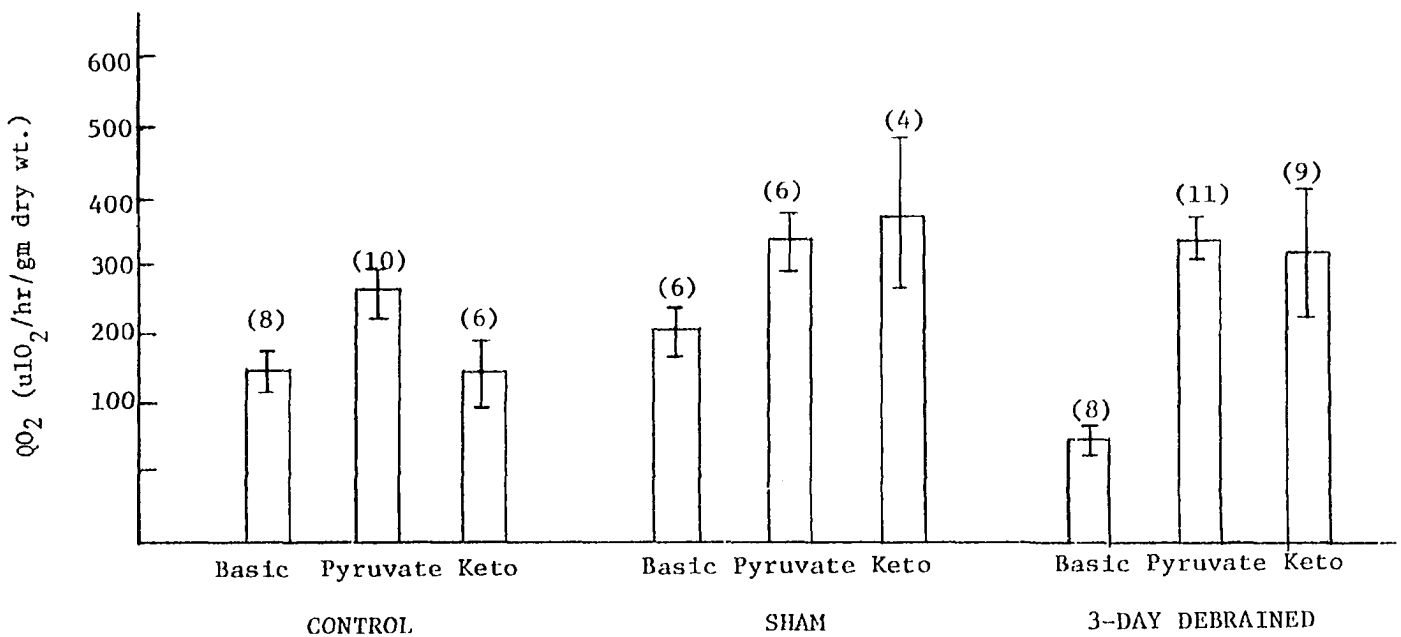
mitochondria from three day debrained worms had a mean value of 0.398 ± 0.114. The mitochondria from the control worms had a slightly lower QO_2 of 0.376 ± 0.099. No significant difference was detected between the means using students t-test.

Manometrically measured oxygen consumption of intestine from control, sham operated and three day debrained worms using key intermediates is summarized in the bar graph (Figure 3). In the basic media the intestinal tissue from control worms had a mean value of 272 $\pm$ 44. The intestine from sham operated worms had a mean QO_2 of 326 $\pm$ 87 and tissue from three day debrained worms had a mean QO_2 of 162 $\pm$ 55.

In basic media plus alpha-ketoglutarate the tissue from control worms had a mean QO_2 of 323 $\pm$ 117. The mean QO_2 of the tissue from sham operated worms was 498 $\pm$ 208 and oxygen quotient of tissue from three day debrained worms was 496 $\pm$ 268.

In basic media containing pyruvate the control tissue had a mean oxygen quotient of 387 $\pm$ 133 while tissue from the sham operated worms had a QO_2 of 457 $\pm$ 119. Statistical comparisons of mean oxygen quotients of control and three day debrained worms are summarized in Table 7. The mean oxygen quotient of the sham operated worms were slightly elevated in all media used. However, no significant difference was detected ($P > .05$) between the sham operated and control. The oxygen quotient of the sham operated worms were determined to assess the effect of the stree of operation on the QO_2 of intestinal tissue. The QO_2 of intestine from three day debrained worms were significantly lower ($P < .05$) compared to control. Addition of either alpha-ketoglutarate or pyruvate to basic media containing tissue from control worms caused a elevation in QO_2 of intestinal tissue but this elevation was not significant ($P > .05$). Addition of pyruvate or alpha-ketoglutarate to the basic media containing intestinal tissue from three day debrained

Figure 3: The effect of key intermediates on the oxygen consumption of intestinal tissue from control, sham operated and three day debrained worms.



Numbers in parenthesis are samples sizes
Horizontal bars represent one standard error

TABLE 7

Manometrically measured oxygen consumption of intestine from control and three day debrained worms using key intermediates.

	QO_2 (ul O_2/hour/gm. dry weight)				
Substrate	Control	(N)	Three day debrained	(N)	Prob.
Basis medium	272 ± 44	(8)	165 ± 55	(8)	P .05
Basic medium+ ketoglutarate	323 ±117	(10)	452 ± 119	(11)	P .05
Basic medium+	387 ±133	(6)	416 ± 276	(9)	P .05

worms resulted in a large increase in QO_2. The increase was significant ($P < .05$) with the addition of either pyruvate or alpha-ketoglutarate. This increase due to the addition of key intermediates elevated the level of the QO_2 to a level comparable to that of the control.

The effect of key intermediates on the QO_2 of intestinal tissue from control and ten day debrained worms is presented in Table 8. The mean of QO_2 of intestinal tissue from ten day debrained worms was higher but not significantly different from the QO_2 of intestinal tissue from control worms. The addition of pyruvate to the basic weight containing control tissue caused an elevation of QO_2 which was not significantly different from the control tissue in basic media. Addition of either intermediate to basic media with tissue from ten day debrained worms resulted in a slight decrease in mean oxygen quotient with both

TABLE 8

The effect of key intermediates on manometrically measured oxygen consumption of intestinal tissue from control and ten day debrained worms.

	QO_2 (ul O_2/hour/gm. dry weight)				
Substrate	Control	(N)	Three day debrained	(N)	Prob.
Basic media	551 $\pm$ 219	(4)	842 $\pm$ 287	(4)	P .05
Basic media^{+} pyruvate	531 $\pm$ 167	(4)	761 $\pm$ 107	(4)	P .05
Basic media^{+} ketoglutarate	742 $\pm$ 306	(4)	754 $\pm$ 326	(6)	P .05

intermediates. In neither case were the decreases significant (P .05).

Polarigraphically determined oxygen quotients from whole intestinal tissue from control and three day debrained worms using key intermediates is shown in Table 9. The tissue from control worms had a significantly greater QO_2 than those of tissue from three day debrained worms when assayed in basic media ($P < .05$). The addition of either key intermediate to the basic media containing intestinal tissue from control worms did not cause a significant effect on the QO_2. Addition of the intermediates to basic media containing tissue from three day debrained worms resulted in a significant decrease in QO_2 with pyruvate used as a substrate.

The effect of key intermediates on the polaragraphically measured

TABLE 9

Polaragraphically determined oxygen consumption of whole intestinal tissue from three day debrained and normal worms using key intermediates.

	QO_2 (uM O_2/hour/gm. dry weight)				
Substrate	Control	(N)	Three day debrained	(N)	Prob.
Basic media	58.68 ± 8.6	(4)	43.37 ± 3.8	(3)	P .05
Basic media + pyruvate	40.74 ± 7.5	(4)	27.31 ± 4.9	(3)	P .05
Basic media + ketoglutarate	55.36 ± 12.7	(4)	35.28 ± 2.6	(3)	P .05

oxygen consumption of intestinal tissue from control and three day debrained worms following a two hour preincubation period are presented in Table 10. When measured in basic media the QO_2 of the intestinal tissue from control worms were significantly higher than the QO_2 of the intestine from three day debrained worms. No significant increase was noted when either pyruvate or alpha-ketoglutarate were added to basic media containing tissue from control worms. A significant increase ($P < .05$) in QO_2 of intestinal tissue from three day debrained worms when either pyruvate or alpha-ketoglutarate were added to the basic media was noted. The increase due to addition of key intermediates brought the level of the QO_2 to a level comparable to the oxygen quotient of the control tissue.

TABLE 10

The effect of key intermediates on polaragraphically determined oxygen consumption of intestinal tissue from normal and three day debrained worms following a two hour incubation period.

	QO_2 (uM O_2/hour/gm. dry weight)				
Substrate	Control	(N)	Three day debrained	(N)	Prob.
Basic media	77.45 ± 15.96	(4)	40.40 ± 12.36	(6)	P .05
Basic media + pyruvate	78.36 ± 1.47	(4)	68.58 ± 3.56	(4)	P .05
Basic media + ketoglutarate	86.73 ± 2.65	(4)	65.15 ± 3.12	(4)	P .05

DISCUSSION

Although the activity of the mitochondria from three day debrained worms had a slightly lower oxidative activity (Table 3) than those from the controls this difference was not significant ($P > .05$). Oxygen consumption of the intestinal mitochondria from three day debrained and control worms was also measured using pyruvate as a substrate. No significant difference was detected between experimental and control giving further indication that the respiratory chain is intact in the mitochondria of three day debrained worms. The results of measuring the respiratory activity indicate that the intestinal mitochondria electron transport enzymes are intact in both three day debrained worms and the decrease in oxidative activity noted in whole tissue from three day debrained worms was not due to a defect in electron transport enzymes.

Direct stimulation of QO_2 of whole intestinal tissue from both three day debrained and control worms had been reported by Papoe (1973) on the addition of whole brain homogenate in a concentration of one brain per mililiter to the bathing media. This elevation of QO_2 could be due to an activation effect on the mitochondrial enzymes. To determine if this was the case, intestinal mitochondria from three day debrained and control worms were incubated with whole brain homogenates in a concentration of two brain per mililiter. No effect of the brain homogenate was noted on mitochondria from control (Table 4) or three day debrained worms (Table 5). The brain homogenate was

prepared by the method of Papoe (1973) and was assumed to be active although the homogenates were not tested for activity during the present experiments. This lack of any significant effect on the mitochonrial oxidative capacity could indicate that there is not a effect on the mitochondria by the neurosecretory from the brain. Keeley and Waddill (1971) also noted that there was no direct effect of corpra cardiaca extract on in vitro succinoxidase activity of mitochondria from thirty day cardiaectomized animals. From the investigation on the mitochondria from intestinal tissue it can be concluded that they remained unchanged in the three day debrained worms. This effect of the lack of neurosecretory factors which cause a depression in the whole tissue oxygen quotient of three day debrained worms was not reflected in the respiratory electron transport system.

In both the insect fat body and the rat liver mitochondria there was an effect on the mitochondria after removal of the hormone responsible for regulation of oxidative metabolism. In both these cases it is possible to have a long term removal of the hormone; by cardiaectomy in the cockroach or thyroidectomy in the rat. The lack of an effect in the intestinal tissue from three day debrained worms may be due to a new source of neurohormone present on or about the third day after debraining. Orenstien (1971) using histochemical techniques has found an increase in neurosecretory activity of cells in the ventral nerve cord occurred on or about the third day post debraining. It is possible that these cells are producing a neurosecretory factor that was produced by the suprapharangeal ganglia

(brain). The presence of this factor from the ventral nerve cord could cause the oxygen consumption to return to normal levels. This correlates well with the observations of Elizinga (1963) for whole body oxygen consumption and for body wall and intestinal tissue (Nelson, 1969). In both of these cases oxygen consumption was seen to increase after reaching a maximum depression at three days after debraining. At about eight to ten days post debraining the oxygen consumption returned to normal or slightly above normal levels for intestinal tissue. Because of some other factor that elevates the QO_2 after three days post debraining it is not possible to have an effect on mitochondria. In both the cockroach and the rat the sole source of hormone can be removed for extended periods and the chronic effect observed. There is evidence that the neurohormone regulating oxidative metabolism exerts its effect by regulating the synthesis of the respiratory enzymes (Keeley, 1972). Since this is a relatively slow process, a long period of withdrawal of the hormone is required. Since in the earthworm only a relatively short term effect was observed,this is a reason no effect was seen on the mitochondria. It is also possible that there is no effect at all of the neurosecretory substance on intestinal mitochondria.

Since the respiratory chain of the mitochondria seemed to be unaffected, the difference that was observed in oxygen consumption between three day debrained and normal worms may be at the glycolytic level. To test the effect of substrates and to further localize the effect, studies of oxygen consumption of intestine from three debrained

and control worms were determined using the key intermediates pyruvate and alpha-ketoglutarate. Results from these determinations (Table 7) confirmed findings of Nelson (1969) and Papoe (1973) that the QO_2 of intestinal tissue from three day debrained worms were significantly lower (162 ± 55) compared to control (272 ± 44). Addition of pyruvate or alpha-ketoglutarate to bathing media containing control tissue caused a general increase in QO_2. This is in agreement with Petrucci (1958) who found alpha-ketoglutarate stimulated oxygen consumption of body wall homogenates of the annelid P. velutinus. Addition of the key intermediates to intestinal tissues from three day debrained worms caused a much greater effect on the oxygen quotient that was seen in controls. Addition of either pyruvate or alpha-ketoglutarate brought the oxygen consumption of the three day debrained worms up to the level of the controls. This effect of key intermediates was opposite to the effects observed by Keeley and Friedman (1968) in fat bodies from thirty day cardiaectomized cockroaches. They observed that addition of key intermediates failed to return the oxygen quotient of the cardiaectomized animal to the level of controls. This failure of key intermediates to return the QO_2 to normal levels when added to fat bodies from thirty day cardiaectomized animals led then to postualte that the mitochondria were the primary site being affected. This was confirmed in the same study by a significant decrease in succinoxidase activity and later (Keeley, 1972) by a reduced level of cytochrome c reductase in fat body mitochondria from thirty day cardiaectomized cockroaches. Since in the intestinal tissue from three day debrained

earthworms there was a significant effect of the key intermediates it would be likely that the mitochondrial electron transport function would be intact in order to provide this increase level of oxidation. Confirmation of this was provided by evidence presented previously that mitochondrial respiratory were unchanged in intestine from three day debrained worms.

In isolated intestine and body wall tissues (Nelson, 1969) it has been observed that the QO_2 began to return to normal after three days post debraining and in the case of excised intestinal tissues reaches above normal levels at about eight to ten days after debraining. If the increase in oxygen consumption, seen in ten day debrained worms, was due to secretion of the ventral nerve cord then addition of key intermediates should have no effect. This was the case in tissues from ten day debrained and control worms. The oxygen quotients of the ten day debrained worms were higher that controls (Table 8). This was in agreement with the observations of Nelson (1969). Addition of either pyruvate or alpha-ketoglutarate caused a slight but insignificant decrease in the QO_2. Also, the difference in response to addition of key intermediates between three day and ten day debrained worms may be due to the presence of neurosecretory substances from the ventral nerve cord in the ten day debrained worms which is able to correct what ever metabolic effect that is caused by absence of the neurosecretory substance in three day debrained worms. Given that the addition of key intermediates to intestine from three day debrained worms returns the QO_2 to normal levels even though

saturation levels of glucose were present, it is possible that the increased QO_2 seen in three day debrained worms is due to a lack of these substrates, a decrease in cell permeability to them or decrease in enzyme levels or activity involving these substrates.

Using the polaragraphic techniques the QO_2 of tissue from three day debrained worms were significantly lower than the control worms (Table 9). This is in agreement with the data from manometric experiments (Table 7). Using the polaragraphic method, however, no increase in QO_2 was noted on addition of key intermediates but a significant decrease was observed. This was a different result than observed with the manometric method. Since in the manometric method the tissue was isolated in the basic media for about two hours prior to determination of the QO_2, it was possible that during that time the tissue became depleted of substrates and will respond to additions. For this reason the QO_2 were measured polaragraphically following incubation of both control and three day debrained tissue in basic media for two hours. Determination made after the two hour incubation period show that the QO_2 of the three day debrained was again significantly lower than the controls (Table 10). Addition of key intermediates following the preincubation did cause a significant increase in oxygen quotient of three day debrained worms but did not significantly affect the control tissue. These results were similar to those obtained with manometric methods. The results from polaragraphic measurements following two hour incubation indicate that the levels of metabolic intermediates in the control and three day debrained intestine may be

different. When the QO_2 was determined with no incubation period there was no increase QO_2 noted on addition of key intermediates in either control or three day debrained because tissue levels of substrates may be at saturation. After preincubation the levels in three day debrained were depleted and thus these tissue responds to addition of key intermediates and if the level of substrate in control were originally higher they would not be depleted below saturation levels during the incubation period.

Dastoli (1964) has presented evidence for the existence of citric acid cycle enzymes in the body wall homogenates of L. terrestris and found them to be similar in function to the mammalian systems. The ability of the intestinal mitochondria used in this investigation to oxidized some key glycolytic and Kreb cycle intermediates were tested in order to gain further evidence for the existence of these enzymes systems (Table 2). Succinate, alpha-ketoglutarate, citrate, malate and pyruvate were all oxidized by the mitochondria. The rate of oxidation of succinate was highest as might be expected because it is tightly bound to membrane and less likely to be lost or damaged during the isolation procedure. The ability of earthworm intestinal mitochondria to oxidized these substrates gives additional evidence that the citric acid cycle enzymes are present in L. terrestris and operated in a similar manner to mammalian systems. The above results as well as the results of inhibitor studies mentioned previously indi0 cate that the earthworm intestinal mitochondria function in similar manner to mammalian mitochondria.

SUMMARY

1. The present study was done in order to determine if depression in oxygen consumption seen in intestinal tissue from three day debrained worms was reflected in mitochondrial oxidative capacity.
2. Mitochondria isolated from non-debrained earthworms intestinal tissue were shown to oxidize succinate, pyruvate, citrate, alpha-ketoglutarate and malate.
3. Succinic acid oxidation by mitochondria from intestinal tissue was inhibited by 10^{-3} M KCN and 10^{-6} M antimyosin indicating that the mitochondria of the earthworm intestinal tissue were at least similar in function to the mammalian system and that they were intact.
4. Succinoxidase activity of intestinal mitochondria from three day debrained and control worms were compared and found not to be statistically different ($P. > .05$).
5. Addition of whole brain homogenate did not have a significant effect ($P > .05$) on the succinoxidase activity of intestinal mitochondria from control on three day debrained worms.
6. Manometric measurement of QO_2 of whole intestinal tissue confirmed observations of Nelson (1969) and Papoe (1973) that the QO_2 of intestinal tissue of three day debrained worms were significantly lower ($P < .05$) than the QO_2 of control tissue.
7. Addition of the key intermediates to basic media containing interestinal tissue from control or three day debrained worms re-

sulted in an significant increase ($P < .05$) in manometrically measured oxygen quotient to a level comparable to control tissue.

8. The oxygen quotients of intestine from ten day debrained worms were higher than control but were not significantly different (P .05). Addition of the key intermediates to basic media containing whole intestinal tissue had no effect ($P > .05$) on the oxygen quotient of either ten day debrained or control.
9. Addition of key intermediates to basic media containing intestinal tissue from control or three day debrained worms resulted in a decreased ($P < .05$) polaragraphically determined QO_2 when determined immediately after excission compared to the control when alpha-ketoglutarate was added to the media. No significant change in QO_2 was noted ($P > .05$) when pyruvate was used as a substrate. However, following a two hour preincubation period in basic media showed the QO_2 of the intestinal tissue from three day debrained worms to be significantly lower ($P < .05$) compared to control tissue. Addition of the key intermediates to media containing the control had no significant effect ($P > 0.5$) on the QO_2.

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