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Oxidative Metabolism in *Ascaris lumbricoides* from the Pig

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The oxygen tension of the pig intestine is thought to be low (Bueding, 1949), and for this reason it has often been doubted whether oxygen is essential to *Ascaris lumbricoides*. Many workers have demonstrated an uptake of oxygen by *Ascaris* and rates up to 500 μl./g. (wet wt.) of whole worm per hr. have been observed in an atmosphere of oxygen (Grembergen, Damme & Vercruysse, 1949). Laser (1944) has shown that prolonged anaerobiosis causes an oxygen debt. Grembergen *et al.* (1949) and Bueding & Charms (1952) have demonstrated a succinoxidase system in preparations of muscle from *A. lumbricoides*, and Bueding & Charms report that this is of an unusual kind, since experiments failed to reveal the existence of a cytochrome system in the tissues.

EXPERIMENTAL

Tissue preparations. Worms were obtained from the slaughter-house. The specimens, after having been washed in warm water, were conveyed to the laboratory in large vacuum flasks containing 'keeping medium' (Baldwin & Moyle, 1947) at 38°. The worms were transferred to fresh medium on arrival at the laboratory and again after 24 hr. They were used on the day of removal from the host or on the succeeding day, except in cases in which it had been shown that activity in preparations from third-day worms was similar to that in first-day preparations.

Whole suspensions of muscle in 0·5% KCl solution were prepared with an homogenizer (Potter & Elvehjem, 1936). A portion (1 ml.) containing 0·4–0·6 g. (wet wt.) of tissue was added to each flask.

A particulate fraction was isolated from muscle by a method based on that of Schneider (1948), 0·2 M sucrose being used. A 20% (wet wt./vol.) suspension of muscle in 0·2 M sucrose was prepared and centrifuged at 600 *g* for 10 min. at 2°. The supernatant was decanted and centrifuged at 18000 *g* for 10 min. in the cold. The residue from about 60 ml. of original suspension was triturated with about 15 ml. of 0·2 M sucrose solution. Such a suspension contained about 0·5–1·0 mg. of N/ml.

Acid-precipitated suspensions were prepared as follows. A 20% (wet wt./vol.) suspension of muscle in 0·05 M phosphate buffer at pH 7·4 was centrifuged at 600 *g* for 10 min. at 2°. The supernatant was adjusted to pH 5·7 with N acetic acid and the precipitate collected immediately by centrifuging at 1000 *g* for 20 min. at 2°. The precipitate was suspended in an equal volume of 0·02 M phosphate buffer at pH 7·4.

Materials. Succinic acid was recrystallized three times from hot water. The succinic acid was dissolved and neutralized, and the sodium salt precipitated with ethanol and recrystallized from 80% ethanol. Citric acid, α-oxoglutaric acid and L-malic acid were commercial products (L. Light and Co. Ltd.). Sodium fumarate and *cis*-aconitic acid were gifts from Dr K. R. Rees. The acids were neutralized with NaOH before use. Diphosphopyridine nucleotide (DPN) was prepared by a method described by LePage (1949). Adenosine triphosphate (ATP) was prepared by the method of Dounce *et al.* (1948). Cytochrome *c* was prepared from beef hearts by the method of Keilin & Hartree (1937). Hexokinase was prepared by the method of Berger, Slein, Colowick & Cori (1946) carried to the second alcohol-precipitation stage. The enzyme was dissolved in 1% glucose and stored at −10°. Catalase prepared from blood by the method of Herbert & Pinsent (1948) was a gift from Miss L. Hopkinson. It was assayed by the method of Goldblith & Proctor (1950). The units were based on the unimolecular reaction constant (Sumner & Somers, 1947).

The experiments were carried out in the Warburg manometric apparatus with flasks of about 20 ml. vol. at 38°, 5 min. being allowed for gassing and 10 min. for equilibration. The gas phase was oxygen, except where otherwise stated, and the volume of fluid in the flasks was 3 ml.

RESULTS

The succinoxidase system

In preliminary experiments the rate of oxidation of succinate by whole suspensions of muscle in 0·5% KCl solution was investigated and found to be

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proportional to the oxygen tension. The rate was markedly reduced by malonate but ATP was without effect. In phosphate buffer pH 7·7 was the optimum for the system and the rate was independent of phosphate concentration over the range 0·0133–0·0667 M.

In order to obtain a convenient uptake of oxygen with whole suspensions of muscle it was necessary to add large quantities to the flasks, and to overcome this difficulty particulate suspensions were prepared and used for most of the investigation.

Table 1. *Effect of oxygen tension and ethylenediaminetetraacetic acid (EDTA) on the succinoxidase system*

Sodium succinate 0·025 M; $MgSO_4$, 0·0066 M; sodium potassium phosphate buffer pH 7·4, 0·0066 M; 1 ml. of a suspension of particles from muscle in 0·2 M sucrose solution; temp., 38°; vol. 3 ml.; duration of experiments, 30 min.

Expt. no.	Description	EDTA (M)	Uptake of oxygen (μl.) In oxygen	In air
1	Complete system	—	61	18
	No succinate	—	Nil	Nil
2	Complete system	—	64	—
	Complete system	0·0013	73	—
	Complete system	0·002	57	—
	Complete system	0·0026	72	—

Such particulate preparations were found to oxidize succinate at a rate corresponding to a $Q_{O_2}^{O_2}$ (N) (μl. O_2/mg. N/hr. in O_2) of 240 (± 12 %) at 38°. The concentration of phosphate buffer at pH 7·4 did not affect the rate, neither did cytochrome *c* over a range of phosphate concentrations. The system was, however, greatly affected by oxygen tension, and results in Table 1 show the succinoxidase system to be at least three times as active in pure oxygen as it is in air. Altmann & Crook (1953) have shown that ethylenediaminetetraacetate (EDTA) stimulates the succinoxidase system in Keilin horse-heart preparations, but in the present preparations EDTA was without effect. DPN and ATP similarly had no effect on the rate of uptake of oxygen by the system.

Laser (1944) has reported that hydrogen peroxide is formed in muscle-pulp preparations from *A. lumbricoides* at high oxygen tensions or in the presence of methylene blue, and he observed increases in the rate of uptake of oxygen when hydrogen peroxide was removed from the reaction medium by a coupled oxidation with ethanol in the presence of catalase. Bueding & Charms (1952) have observed a similar increase in the uptake of oxygen in the presence of ethanol and catalase when succinate was the substrate, and increases were obtained in the present study with isolated particles (Table 2).

As a result of the coupled oxidation, ethanol is converted into acetaldehyde, and Table 3 shows that semicarbazide placed in the centre well, by removing this substance from the reaction medium, brings about an increase in the rate of uptake of oxygen. Further increases may be brought about by raising the pH to 8·0 and by adding Mn^{2+} ions (Table 3). These findings confirm earlier work of Bueding & Charms (1952) on muscle-pulp preparations.

The addition of methylene blue to the fortified particulate suspension was found to result in a well-marked increase in the rate of oxygen uptake (Fig. 1). Ferricyanide added as hydrogen acceptor

Table 2. *Effect of catalase and ethanol on the succinoxidase system*

Sodium succinate, $MgSO_4$ and phosphate buffer as in Table 1; 1 ml. of a particulate suspension from muscle in 0·2 M sucrose solution; additions as indicated; temp., 38°; duration of experiments, 60 min.

			Uptake of oxygen (μl.)			
			Expt. 1		Expt. 2	
Description	Catalase units	Ethanol (M)	0–30 min.	30–60 min.	0–30 min.	30–60 min.
Complete system	—	—	33	24	47	35
Complete system	5·8	—	23	27	—	—
Complete system	—	4×10^{-2}	23	23	—	—
Complete system	5·8	4×10^{-2}	37	37	55	46
No succinate	5·8	4×10^{-2}	Nil	Nil	Nil	Nil

Table 3. *Effect on the succinoxidase system of* pH, Mn^{2+} *and semicarbazide*

Sodium succinate, 0·025 M; $MgSO_4$, 0·0066 M; catalase, 5·8 units; ethanol, 0·04 M; sodium potassium phosphate buffer, 0·0067 M; other additions as indicated. 1 ml. of a suspension of particles from muscle in 0·2 M sucrose; temp., 38°; duration of experiment, 1 hr.

pH	$MnCl_2$ (M)	Semicarbazide in centre well (M)	Uptake of O_2 (μl.)
7·4	—	—	27
8·0	—	—	36
8·0	1×10^{-3}	—	55
8·0	1×10^{-3}	0·2	67

in absence of oxygen was shown similarly to allow of a greater rate of oxidation. The disproportion in the rates of uptake of oxygen in the presence and absence of methylene blue encouraged the view that the natural hydrogen-transporting system considerably limits the rate of oxidation of succinate in *A. lumbricoides*.

Since cytochrome oxidase plays an important part in terminal oxidation in most organisms an attempt was made to demonstrate its presence by the method of Potter (1949). In confirmation of the results of Bueding & Charms (1952) it was not possible to detect cytochrome oxidase activity in the particulate suspensions. Cyanide is known to be a powerful inhibitor of cytochrome oxidase. In the present study, however, potassium cyanide at a final concentration of 0·01 M brought about a striking increase in the rate of uptake of oxygen by the succinoxidase system (Table 4).

Although EDTA had no effect on the uptake of oxygen in the absence of methylene blue, an appreciable increase in the rate resulted from adding EDTA in the presence of the dye (Fig. 1).

Again, in the system with methylene blue, DPN brought about a well-marked increase in the rate of uptake of oxygen. DPN had a similar effect on a system in which an acid-precipitated suspension, as described by Keilin & Hartree (1940), was used.

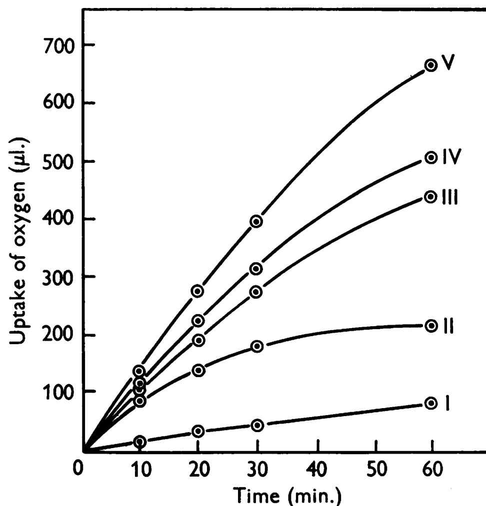

Fig. 1. Effect of methylene blue on the succinoxidase system. Sodium succinate, 0·025 M; sodium potassium phosphate buffer pH 7·4, 0·0066 M; $MgSO_4$, 0·0066 M; nicotinamide, 0·04 M. Additions: DPN, 0·00033 M; EDTA, 0·001 M; methylene blue, 0·033 %; 1 ml. of a suspension of particles from muscle in 0·2 M sucrose. Vol., 3 ml. I, Complete system; II, complete system + methylene blue; III, complete system + methylene blue + DPN; IV, complete system + methylene blue + EDTA; V, complete system + methylene blue + DPN + EDTA. There was no endogenous oxygen uptake.

Tricarboxylic acid-cycle enzymes in a particulate fraction from muscle

Several intermediates in the tricarboxylic acid cycle were found to support an uptake of oxygen when added to fortified particulate suspensions from muscle (Table 5). The rates were much greater in the presence than in the absence of methylene blue. Like succinate, α-oxoglutarate, fumarate and L-malate were rapidly oxidized in the system, but no uptake of oxygen was observed in the presence of pyruvate, citrate, *cis*-aconitate, oxaloacetate, or pyruvate and oxaloacetate together. Addition of TPN did not promote oxidation of citrate, and omission of DPN had a negligible effect on the oxidation of L-malate and α-oxoglutarate. No uptake resulted from 3-phosphoglyceric acid or from sodium acetate in the presence of catalytic amounts of L-malate.

Oxidative phosphorylation

In a particulate suspension fortified with Mg^{2+}, ATP, DPN, glucose and hexokinase in phosphate buffer at pH 7·4 no phosphorylation was observed as a result of the oxidation of either α-oxoglutarate or succinate. Fluoride and EDTA failed to elicit phosphorylation, neither was any observed when

Table 4. *Effect of cyanide on the succinoxidase system*

Succinate, $MgSO_4$ and phosphate buffer as in Table 1; 1 ml. of a suspension of particles from muscle in 0·2 M sucrose solution; neutralized KCN added where indicated. In flasks containing cyanide 0·1 ml. of 2 M-KCN and 0·1 ml. of 0·002 M-KOH were placed in the centre well; temp., 38°.

Expt. no.	Description	KCN (M)	Uptake of oxygen (µl.)		
			0–10 min.	0–30 min.	0–60 min.
1	Complete system	0·01	18	—	96
	Complete system	—	5	—	44
	No succinate	0·01	Nil	—	Nil
	No succinate	—	Nil	—	Nil
2	Complete system	0·01	27	70	—
	Complete system	—	14	49	—
	No succinate	0·01	Nil	Nil	—
	No succinate	—	Nil	Nil	—

Table 5. *Oxidation of some intermediates in the tricarboxylic acid cycle in the presence and absence of methylene blue*

Sodium potassium phosphate buffer pH 7·4, 0·0067 M; $MgSO_4$, 0·0067 M; DPN, 0·00033 M; nicotinamide, 0·04 M; ATP, 0·001 M; substrate as indicated; methylene blue added where indicated; 1 ml. of a suspension of particles in 0·2 M sucrose from muscle; temp., 38°; duration of experiments, 30 min.

Expt. no.	Substrate (0·01 M final concn.)	Methylene blue (g./100 ml.)	Uptake of O_2 (µl.)
1	α-Oxoglutarate	0·033	176
	α-Oxoglutarate	—	66*
	Fumarate	0·033	181
	L-Malate	—	94†
	cis-Aconitate	0·033	Nil
	No substrate	0·033	Nil
2	Citrate	0·033	Nil
	L-Malate	0·033	225
	α-Oxoglutarate	0·033	246
	No substrate	0·033	Nil

* $Q_{O_2}^{O_2}$ (N), 165. † $Q_{O_2}^{O_2}$ (N), 235.

the particles were isolated in the EDTA–sucrose medium described by Cleland & Slater (1953). Extensive esterification of phosphate occurred, however, when mitochondria from rat liver were incubated under similar conditions.

By including catalase and perienteric fluid in the reaction medium Chin & Bueding (1954) were able to demonstrate oxidative phosphorylation both in whole homogenates and in particulate fractions from the muscle of *A. lumbricoides*. These workers are of the opinion that the perienteric fluid contributes both the substrate and an undiffusible component required for the process.

In Table 6 are shown the results of some preliminary experiments in which catalase and ethanol, together with perienteric fluid, were present in the system. It is seen from Expt. 1 that a small esterification of phosphate occurred and that the esterification was greatest when perienteric fluid provided the substrate. Succinate caused a reduction, as did EDTA, and succinate by itself did not support oxidative phosphorylation. In Expt. 2, pyruvate seemed only slightly to affect the degree of phosphate esterification in the presence of perienteric fluid. Pyruvate alone did not give rise to phosphate uptake, neither did α-oxoglutarate, although the rate of uptake of oxygen in the presence of α-oxoglutarate was relatively great.

DISCUSSION

It is unusual that the uptake of oxygen by homogenates and particulate suspensions should increase several times with increase in the oxygen tension. The phenomenon has been observed in segments of whole worm by Harnisch (1933), in whole worm and in muscle pulp by Laser (1944), and in muscle pulp by Bueding & Charms (1952). A similar dependence of oxygen consumption upon oxygen tension has been found in certain small nematode parasites of the intestinal tracts of sheep and rat (Rogers, 1949).

DPN and ATP were found to have no effect on the rate of uptake of oxygen, but although ATP was without effect in the presence of methylene blue DPN brought about a marked increase. An increase with DPN was observed when a preparation of muscle similar to that used by Keilin & Hartree (1940) in their classical experiments with the succinoxidase system was employed. It has not been possible to isolate a one-step oxidation of succinate to the fumarate–malate complex, and consequently the point of action of DPN cannot for the present be defined.

Table 6. *Oxidative phosphorylation in a particulate fraction isolated from the muscle of* A. lumbricoides

Glucose, 0·01 M; ATP, 0·001 M; hexokinase solution, 0·05 ml.; nicotinamide, 0·04 M; DPN, 0·00033 M; sodium phosphate buffer pH 7·4, 0·0016 M; tris (aminotrishydroxymethylmethane) buffer pH 7·4, 0·027 M; NaF, 0·013 M; catalase, 6 units; ethanol, 0·04 M; KCl, 0·025 M; 0·5 ml. of a suspension of particles in 0·2 M sucrose. Final concn. of substrate (when present): succinate 0·025 M; pyruvate 0·01 M; α-oxoglutarate 0·01 M. Temp., 38°; duration of experiments, 2 hr.

Expt. no.	Substrate	Perienteric fluid (ml.)	EDTA (M)	P uptake (µmole) In N_2	P uptake (µmole) In O_2	O_2 uptake (µatoms)	P:O quotient
1	Succinate	0·3	0·001	Nil	0·32	3·5	0·09
	Succinate	0·3	—	Nil	0·45	3·1	0·15
	Succinate	—	0·001	Nil	Nil	1·7	—
	Succinate	—	—	Nil	Nil	3·6	—
	—	0·3	0·001	Nil	0·45	1·5	0·30
	—	0·3	—	Nil	0·74	2·4	0·31
2	Pyruvate	0·3	—	Nil	0·71	2·0	0·35
	—	0·3	—	Nil	0·52	2·0	0·26
	Pyruvate	—	—	Nil	Nil	Nil	—
	—	—	—	Nil	Nil	Nil	—
	α-Oxoglutarate	0·3	—	Nil	0·23	2·2	0·10
	α-Oxoglutarate	—	—	Nil	Nil	9·7	—

Potassium cyanide, in contrast with its effect on the mammalian succinoxidase system, brought about a marked increase in the rate of uptake of oxygen. There is a possibility that cyanide, because of its ability to combine with ketonic substances, reacts with oxaloacetic acid, so relieving the inhibition which this acid imposes on succinic dehydrogenase (Pardee & Potter, 1948) and thereby masking a possible overall inhibitory effect. However, since the rate of uptake of oxygen in the flasks containing cyanide is twice that of the controls even at the end of an hour, it is doubtful if this explanation is the right one. The increase may follow from a combination of cyanide with certain iron porphyrins. It is known that the oxidation–reduction potentials of cyanide haemochromogens are lower than those of haemochromogens in which nitrogenous substances other than cyanide are the substituents (Barron, 1937). Haemochromogens may thus be brought into favourable oxidation–reduction relationship with the hydrogen-transporting system thereby allowing increased oxidation of the substrate. The behaviour of the porphyrin of the body-wall haemoglobin of *Ascaris* (Davenport, 1949) may be mentioned in this connexion.

Although the bulk of the evidence suggests that a cytochrome system, is absent from *A. lumbricoides*, Grembergen *et al.* (1949), using Latapie breis, have obtained evidence for the existence of a cytochrome oxidase with typically mammalian characteristics.

Of the intermediates in the tricarboxylic acid cycle, α-oxoglutarate, succinate, fumarate and L-malate were oxidized in the system described. Since carbohydrate was shown *in vitro* to be broken down anaerobically only as far as phosphoglyceric acid (Rathbone & Rees, 1954), it is difficult to imagine how the anaerobic and aerobic pathways work together in cellular metabolism.

It may be significant that *in vitro* no hydrogen acceptor is produced for the reoxidation of reduced DPN formed in glycolysis. There is a possibility that oxygen enters directly into the scheme at this point, and a condition similar to that found in *Plasmodium gallinaceum* and *Trypanosoma evansi* (Marshall, 1948*a*, *b*) results. Alternatively, if oxaloacetic acid is able to act as a hydrogen acceptor *in vivo*, then the system oxaloacetate$\rightleftharpoons$malate might function as a hydrogen-carrier system, as suggested by Szent-Györgyi (1935), who found that in muscle the reversal of dehydrogenation of malate took place with unusual rapidity. Krebs (1943) has affirmed the possibility that this reaction is of some importance as a hydrogen-carrier system.

The present study was undertaken with a view to the elucidation of enzyme systems that involve oxygen, and to provide a basis for speculation on the possibilities of parasitic adaptation at the cellular level.

SUMMARY

1. A succinoxidase system was found to be present in a particulate fraction isolated from the muscle of *A. lumbricoides*. Rates of uptake of oxygen averaged 240 μl./mg. of N/hr. at 38° in an atmosphere of oxygen. The rate in pure oxygen was about three times that in air.

2. In an atmosphere of pure oxygen, addition of methylene blue to the succinoxidase system caused a marked increase in the rate of uptake of oxygen.

3. In the presence of methylene blue the addition of ethylenediaminetetraacetic acid and DPN brought about increases in the rate.

4. α-Oxoglutarate, fumarate and L-malate, like succinate, supported an uptake of oxygen when added to fortified particulate suspensions. The rates were appreciably greater in the presence of methylene blue than in its absence. Citrate, *cis*-aconitate, oxaloacetate, and pyruvate with or without oxaloacetate, failed to cause uptake of oxygen.

5. In preliminary experiments no evidence of a typical cytochrome oxidase was obtained.

6. A small amount of oxidative phosphorylation was found to occur in suitably fortified particulate suspensions in the presence of perienteric fluid from *A. lumbricoides*.

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Chitinase in some Basidiomycetes

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The decomposition of chitin by bacteria has been the subject of sporadic interest, but its breakdown by fungi has attracted little interest. Chitin may be assumed to be one of the polysaccharides that are of quantitative importance in the complex series of microbiological syntheses and analyses that occur in any natural soil. Since, like cellulose, chitin is an insoluble polymer of low hydration built up of closely apposed oriented chains, it is likely to be relatively resistant to enzymic breakdown (Tracey, 1953). Most of the fungi contain chitin, and it occurs also in most groups of the soil fauna save perhaps the Protozoa. As a relatively abundant material, probably only slowly broken down enzymically, it may prove of importance as a constituent of the total organic matter of the soil and, even more, of the non-living fraction. Chitin is normally estimated by total hydrolysis followed by determination of glucosamine and sometimes of acetic acid. The method is not specific even if preceded by extensive purification of the material (a process that often includes alkali treatment with risk of deacetylation). Glucosamine is broken down to a variable extent during acid hydrolysis and in crude preparations may arise from substances other than chitin. The use of enzymic hydrolysis should serve as a useful check on chemical methods, for it has the advantage that the end product is acetylglucosamine and any doubt about the acetylated form of the original material is removed. The present work was done in the hope of locating a useful source of chitinase for analytical work (Tracey, 1955). Liquids of considerable chitinase activity have been found to be produced by a number of species of *Lycoperdon*. The fungi are abundant at the beginning of autumn and the enzyme solutions obtained from them are stable for considerable periods.

METHODS

Fungal extracts. Basidiomycete fructifications were minced, and the liquid was then expressed through cloth and centrifuged. Some fungi gave on mincing a gelatinous mass from which it was impossible to express an extract by hand. With these minces a cloth-lined basket centrifuge gave an extract with little trouble. Many *Lycoperdon* species, if harvested at a suitable stage of maturity, contain free liquid which usually accounts for 70–75% of the fresh weight. These fructifications were torn open and spun on the basket centrifuge without mincing. A further extract of considerably higher solid content could be got by mincing the residue from the first treatment.

Chitosan. This is a product of variable composition prepared by the partial deacetylation of chitin (see, for example, Darmon & Rudall, 1950). Lobster (*Homarus*) chitin was heated with 40% (w/v) NaOH solution in a flask fitted with a Bunsen valve in an oven at 105° until the material was soluble in dilute acetic acid. Excess of NaOH solution was decanted after centrifuging, the chitosan dissolved in dilute acetic acid and dialysed against distilled water to give a solution of the salt, chitosan acetate.

Chitin. Cuttlefish (*Sepia*) 'bones' were soaked in dil. HCl until all $CaCO_3$ had been removed. The chitinous laminae were separated from sand and dispersed in cold conc. HCl. As soon as the solution appeared substantially complete the liquid was centrifuged and the supernatant poured into a large volume of distilled water with mechanical stirring. The precipitated chitin was washed repeatedly by decantation and residual material of low molecular weight removed by dialysis against distilled water. The chitin was stored as