OCCURRENCE OF OXIDATIVE PHOSPHORYLATIONS IN THE MUSCLE OF *ASCARIS LUMBRICOIDES*[*]

by

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While oxidative phosphorylations have been studied extensively in certain mammalian tissues, yeast and bacteria, little is known about these processes in invertebrate metazoa, and no information is available indicating whether oxidative phosphorylations occur in helminths. Many of these parasites live in the intestinal tract where the oxygen tension is low. The dependence of parasitic worms on respiratory metabolism for their survival and reproduction varies from one species to another and has no apparent relationship to the oxygen tension of their habitat[1]. Among helminths, *Ascaris lumbricoides* survives for relatively long periods under anaerobic conditions[2,3], and the physiological significance of oxidative metabolism for survival and reproduction of this parasite is not understood. One of the possible functions of biological oxidations in the tissues of *Ascaris* may be the generation of energy-rich phosphate bonds. A study of this problem, reported in the present paper, has revealed the occurrence of oxidative phosphorylations in *Ascaris* muscle.

MATERIALS AND METHODS

Muscle strips of *Ascaris lumbricoides* were obtained essentially in the same manner as described by LASER[4]. The muscle strips were removed carefully from the cuticle with least possible damage, and cooled in a beaker surrounded by chopped ice.

For the preparation of homogenates the muscle strips were washed twice with cold 0.9% KCl solution, and homogenized in two volumes of 0.9% KCl at 2–4° C in an all-glass Potter-Elvehjem homogenizer. The pH of the homogenate was carefully adjusted to 7.0 from time to time during the homogenization.

A particulate fraction was prepared as follows. The muscle strips were homogenized in 9 volumes of a cold sucrose solution (0.25 *M*) at 2–4° with the pH maintained at 7.0–7.3. The homogenate was then centrifuged in a refrigerated centrifuge at 2,000–2,500 r.p.m. for 5 minutes. The supernatant was re-centrifuged at 12,000–13,500 r.p.m. for 10–15 minutes. The second residual fraction was then suspended in the sucrose solution (about 7 to 8 ml for every 10 g of fresh muscle).

In either case the preparation was pipetted immediately into ice-cooled Warburg flasks for the measurement of oxygen uptake. The center well of the flask contained 0.1 ml of 5 *N* KOH on strips of filter paper. The period between the beginning of the muscle dissection and the start of the manometric measurements usually was less than one hour.

The perienteric fluid was pressed out gently with the aid of forceps after both ends of the worms were cut off. The fluid was then centrifuged at a moderate speed (2,500 r.p.m.) for 15 minutes at 2° and the supernatant was used. In a number of experiments, this supernatant was dialyzed

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at 2–4° in a rocking dialyzer for 3 hours against 0.04 *M* tris (hydroxylmethyl)-aminomethane buffer (pH 7.3) which ran through the dialyzer continuously. The volume of the dialyzed solution was approximately the same as that of the fluid before dialysis. Perienteric fluid dialysate was obtained by dialyzing the fluid against an equal volume of glass-distilled water overnight in a refrigerator.

Yeast hexokinase was prepared according to the method of BERGER *et al.*[5] (Step 5). Catalase and DPN* were commercial products (Armour and Pabst, respectively). A solution of the barium salt of ATP (in 0.5 *N* HCl) was converted into the potassium salt by running it through a cation exchange resin (Dowex-50) column and neutralized with KOH. *α*-Lipoic acid was kindly supplied by Dr. I. C. GUNSALUS. Potassium pyruvate was crystallized from pyruvic acid as described by KORKES *et al.*[6]. Nitrogen was purified by passing this gas through a red-hot copper gauze. It has been demonstrated that hydrogen peroxide is produced by respiring *Ascaris* tissues[4]. Furthermore, aerobic phosphorylations by preparations of *Ascaris* muscle were reduced considerably in the absence of added catalase. Therefore, this enzyme was added to the reaction mixture in all experiments.

All incubations were carried out at 25° C. At zero time and at the end of the experimental period the reaction mixtures were de-proteinized by tipping TCA (100% solution) from the side-arm into the main compartment of the vessels (final concentration: 10%). They were then removed from the bath, cooled in ice and centrifuged at 10,000 r.p.m. for one hour at 2–6° C. Supernatants were assayed for inorganic phosphate by the method of FISKE AND SUBARROW[7]. For determination of the total phosphorus content the samples were incinerated first with a H_2SO_4 HNO_3 mixture according to LOHMANN[8].

RESULTS

Uptake of inorganic phosphate by the homogenate of Ascaris muscle

Homogenates of washed muscle strips of *Ascaris lumbricoides* take up inorganic phosphate during the oxidation of endogenous substrate, resulting in P/O ratios of 1.31 to 3.55 (Table I). Anaerobically, the disappearance of inorganic phosphate was considerably lower and amounted, at the most, to one-third of the phosphate taken up aerobically. The addition of undialyzed perienteric fluid to the phosphorylating system increased the rate of respiration but reduced the uptake of phosphate. This increased rate of respiration, however, diminished considerably without a corresponding decline in the uptake of phosphate when a gas phase of air was used instead of pure oxygen. Apparently, oxidation of a substrate or of substrates occurred, which was not related to oxidative phosphorylations and which was more pronounced in pure oxygen than in air.

The inorganic phosphate taken up was recovered predominantly in the difficultly hydrolyzable acid soluble fraction (Table II), indicating the formation of hexose phosphates. There was also some increase in the acid-labile phosphate fraction.

Attempts to remove endogenous substrate from the muscle particles by homogenizing the muscle in a large volume of a solution of potassium chloride (0.9%) and then centrifuging the particulate fraction were unsuccessful. When a solution of sucrose (0.25 *M*) was used, it was found that a particulate fraction, obtained by means of differential centrifugation, showed a very low rate of respiration and no uptake of inorganic phosphate or, in some cases, even a slight increase in inorganic phosphate (Table III). However, oxidative phosphorylations occurred when both DPN and perienteric fluid were added to the system. The relatively low rates of phosphate uptake and the great variations in the P/O ratios in different experiments cannot be explained readily. Possibly, some endogenous substrate, present in various concentrations in different preparations, is oxidized and this reaction may not be associated with phos-

* The following abbreviations will be used throughout: diphosphopyridine nucleotide — DPN
adenosine triphosphate — ATP
trichloracetic acid — TCA

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TABLE I

AEROBIC PHOSPHATE UPTAKE BY HOMOGENATE OF WASHED *Ascaris* MUSCLE

Each vessel contained the following per ml: 0.4 ml muscle homogenate, 0.1 ml phosphate buffer (0.1 *M*, pH 7.4), 0.1 ml fluoride (0.33 *M*), 0.075 ml tris (hydroxylmethyl)-aminomethane buffer (0.4 *M*, pH 7.4), 0.06 ml $MgCl_2$ (0.1 *M*), 0.03 ml glucose (1 *M*), 0.02 ml yeast hexokinase (1:20), 0.01 ml ATP (K-salt, $9.3 \cdot 10^{-2}$ *M*), 0.05 ml catalase (5 %), 0.1 ml TCA (100 %) in side bulb and tipped into the reaction mixture at the beginning or the end of the experiment in each series. Total volume: 2.0 ml (experiments 2 and 3) or 1.0 ml (experiments 1 and 4). Temperature: 25°. Equilibration period: 5 minutes.

Expt. No.	*Time min*	*Addition*	*Gas phase*	*P-uptake μ-atoms*	*O_2-uptake μ-atoms*	*P/O ratio*	*Remarks*
1	30	—	O_2	0.42*	0.22	1.91	low P-concn.**
	30	—	N_2	—0.03	—	—	
2	30	—	O_2	1.62*	0.62	2.62	
	30	—	N_2	0.40	—	—	
3	30	—	O_2	3.46*	2.64	1.31	
	30	—	N_2	1.00	—	—	
	30	perienteric fluid	O_2	2.06*	3.36	0.61	
	30	(0.1 ml/ml)***	N_2	0.95	—	—	
4	10	—	O_2	0.57*	0.16	3.55	
	10	—	N_2	0.21	—	—	
	10	perienteric fluid	O_2	0.63*	0.53	1.18	
	10	(0.15 ml/ml)***	air	0.57*	0.28	2.05	
	10	—	N_2	0.15	—	—	

* Corrected by subtracting the anaerobic phosphate uptake.

** Only 1 μM/ml of phosphate was added to the reaction mixture, while 10 μM/ml of phosphate was introduced in the remaining experiments.

*** Perienteric fluid was centrifuged at a moderate speed (2,500 r.p.m.) for 15 minutes at 0° C.

TABLE II

PHOSPHATE BALANCE AFTER AEROBIC INCUBATION OF *Ascaris* MUSCLE HOMOGENATE

Each vessel had the same composition as listed in Table I. Total volume: 1.0 ml. Temperature: 25°. Incubation period: 30 minutes (after 5 minutes equilibration). All values are expressed in μM of P.

Addition	*Time (min)*	*Inorg.-P*	*7′ P (100° C; 1 N HCl)*	*180′ P (100° C; 1 N HCl)*	*Acid resist. P*	*Total P*
Perienteric fluid (0.1 ml)*	0	10.10	0.60	4.42	6.23	21.4
	30	7.78	1.42	3.41	9.21	21.8
	Difference	—2.32	+0.82	—1.01	+2.98	+0.4
None	0	10.63	0.02	4.18	5.83	20.7
	30	8.88	0.37	3.82	7.13	20.2
	Difference	—1.75	+0.35	—0.36	+1.30	—0.5

* See footnote Table I.

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TABLE III

AEROBIC PHOSPHATE UPTAKE BY A PARTICULATE FRACTION OF *Ascaris* MUSCLE

The *complete system* had the following constituents per ml: particulate fraction: 0.4 ml; 0.11 *M* $MgCl_2$: 0.06 ml; 1.1 *M* glucose: 0.03 ml; 0.44 *M* tris (hydroxymethyl)-aminomethane buffer (pH 7.4): 0.06 ml; 0.055 *M* potassium phosphate buffer (pH 7.4): 0.06 ml; catalase (10%): 0.02 ml; yeast hexokinase (1:20): 0.02 ml; 0.044 *M* KATP: 0.02 ml; 0.33 *M* fluoride: 0.025 ml; 0.08 *M* DPN: 0.03 ml.

Temperature: 25°. Equilibration period: 5 minutes. Duration of experiment: 30 minutes.

Expt. No.	*System*	*Additions*	*Gas phase*	*P-uptake μ-atoms*	*O_2-uptake μ-atoms*	*P/O ratio*
1	Complete	Perienteric fluid (0.1 ml)	O_2	1.09	0.70	1.56
	Complete	Perienteric fluid (0.1 ml)	N_2	—0.24	—	—
	No particulate fraction	Perienteric fluid (0.1 ml)	O_2	nil	nil	—
2	Complete	Perienteric fluid (0.1 ml)	O_2	0.48*	0.80	0.60
	Complete	Perienteric fluid (0.1 ml)	N_2	0.02	—	—
	No fluoride	Perienteric fluid (0.1 ml)	O_2	0.52*	1.02	0.51
	No fluoride	Perienteric fluid (0.1 ml)	N_2	0.17	—	—
3	Complete	Perienteric fluid (0.1 ml)	O_2	1.90	1.70	1.18
	No hexokinase	Perienteric fluid (0.1 ml)	O_2	—0.77	1.40	—
	No DPN	Perienteric fluid (0.1 ml)	O_2	—0.90	1.43	—
	Complete	None	O_2	—0.30	nil	—
4	Complete	Perienteric fluid (0.1 ml)	O_2	0.42	0.32	1.31
	Complete	Dialyzed perienteric fluid (0.1 ml) + perienteric fluid dialysate (0.1 ml)	O_2	0.68	0.46	1.48
	Complete	Dialyzed perienteric fluid (0.1 ml) + succinate**	O_2	0.13	3.83	0.03
	Complete	Perienteric fluid dialysate (0.1 ml)	O_2	—0.32	1.50	—

* Corrected by subtracting the anaerobic phosphate uptake.
** Final concentration: 0.01 molar.

phorylation. Alternatively, some factor required for oxidative phosphorylation varies from one experiment to another, either because of varying concentrations in the worms or because of unavoidable differences in the preparatory manipulations. In the absence of fluoride there was an appreciable anaerobic uptake of phosphate, which was abolished when fluoride was present. Furthermore, it was found that the perienteric fluid not only contributed the substrate, but also an undialyzable component required for oxidative phosphorylation (Tables III and IV).

Attempts were made to determine whether the dialyzable component of the perienteric fluid, required for aerobic phosphorylation, could be replaced by an exogenous substrate. Observations recorded in Table IV indicate that addition of pyruvate also resulted in the uptake of inorganic phosphate. Likewise, the addition of α-ketoglutarate and ethanol produced some disappearance of inorganic phosphate. Succinate, on the other hand, reduced aerobic phosphorylations (Tables III and IV). Cocarboxylase and α-lipoic acid, added either alone or together, had no effect on phosphate and oxygen uptakes of the system. Dinitrophenol, in a final concentration of $3 \cdot 10^{-5}$ *M*, uncoupled completely phoshorylation from oxidation (Table V). The same effect also was observed in the muscle homogenate. However, with higher concentrations of dinitrophenol

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($8 \cdot 10^{-5}$ *M* and upwards), this inhibitory effect was less pronounced, and in some cases (experiments 3, 4 and 6, Table V) an increase in the aerobic phosphate uptake was observed.

TABLE IV

AEROBIC PHOSPHATE UPTAKE BY A PARTICULATE FRACTION IN THE PRESENCE OF PYRUVATE AND DIALYZED PERIENTERIC FLUID

In addition to the constituents listed on Table III, the complete system contained pyruvate (final concentration: 0.02 molar) and dialyzed perienteric fluid (0.1 ml). Total volume: 1 ml. Temperature: 25°. Equilibration period: 5 minutes.

Expt. No.	*System*	*Incubation period (min)*	*Gas phase*	*P-uptake μ-atoms*	*O_2-uptake μ-atoms*	*P/O ratio*
1	Complete	30	O_2	0.38	0.96	0.40
	No dialyzed perienteric fluid	30	O_2	0.09	0.59	0.14
	No pyruvate	30	O_2	nil	nil	—
	Complete	30	N_2	nil	—	—
2	Complete	25	O_2	0.32	0.84	0.38
	Complete	25	air	0.35	0.62	0.57
	No DPN	25	O_2	nil	0.89	—
	Complete	25	N_2	nil	—	—
3	Complete	25	air	0.60	1.02	0.59
	No pyruvate	25	air	nil	0.28	—
	No DPN	25	air	nil	0.96	—
	No dialyzed perienteric fluid	25	air	0.06	0.70	0.09
4	Complete	10	air	0.25	0.17	1.45
	Complete	25	air	0.23	0.38	0.60
	Complete	45	air	0.28	1.05	0.27
5	Complete	10	air	0.37	0.24	1.55
	Complete + succinate (final concn.: 0.02 molar)	10	air	0.19	0.47	0.40
6	Complete	5	air	0.06	0.21	0.29
	Complete	10	air	0.12	0.35	0.34
	Complete	20	air	0.25	0.69	0.36

DISCUSSION

ROGERS AND LAZARUS have reported the uptake of inorganic phosphate by brei or extracts of *Ascaris* muscle[9]. The conditions of their experiments strongly suggest that organic phosphate esters were formed as a result of anaerobic reactions which may be coupled with the action of glycolytic enzymes; the occurrence of the latter in *Ascaris* muscle has been demonstrated[10]. Our results supply confirmatory evidence for the anaerobic esterification of inorganic phosphate by *Ascaris* muscle. In addition, we have observed aerobic phosphorylations by preparations of *Ascaris* muscle, under conditions in which anaerobic phosphorylations proceed at a much slower rate or are not demonstrable at all. As with certain mammalian tissues, such as kidney or liver, oxidative phosphorylations in *Ascaris* muscle are catalyzed by a particulate fraction and are associated with the transfer of electrons or of hydrogen through DPN which is reduced as a result of the oxidation of a substrate, such as pyruvate. On the other hand, oxidative

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TABLE V

EFFECT OF DINITROPHENOL ON AEROBIC PHOSPHORYLATIONS BY PREPARATIONS OF *Ascaris* MUSCLE

In experiment No. 1 the complete system listed in the legend of Table I, in all other experiments the system described in Table IV was used.

Expt. No.	*Muscle preparation*	*Concentration of dinitrophenol (molar)*	*Incubation period (min)*	*Gas phase*	*P-uptake μ-atoms*	*O_2-uptake μ-atoms*	*P/O ratio*
1	Homogenate	—	10	air	2.10	0.53	3.95
		$3 \cdot 10^{-5}$	10	air	—0.39	0.56	—
		—	10	N_2	—0.27	—	—
		$3 \cdot 10^{-5}$	10	N_2	—0.32	—	—
2	Particulate fraction	—	25	air	0.23	0.38	0.60
		$3 \cdot 10^{-5}$	25	air	nil	0.82	—
		$8 \cdot 10^{-5}$	25	air	0.20	1.08	0.19
3	Particulate fraction	—	10	air	0.12	0.35	0.34
		$3 \cdot 10^{-5}$	10	air	nil	0.59	—
		$8 \cdot 10^{-5}$	10	air	0.39	0.36	1.08
4	Particulate fraction	—	25	air	0.60	1.02	0.59
		$3 \cdot 10^{-5}$	25	air	nil	1.36	—
		$1 \cdot 10^{-4}$	25	air	1.17	1.53	0.77
5	Particulate fraction	—	10	air	0.36	0.29	1.24
		$3 \cdot 10^{-5}$	10	air	nil	0.38	—
		$8 \cdot 10^{-5}$	10	air	0.28	0.34	0.82
		—	25	air	0.36	0.78	0.46
		$3 \cdot 10^{-5}$	25	air	nil	0.98	—
		$8 \cdot 10^{-5}$	25	air	0.19	0.86	0.22
6	Particulate fraction	—	10	air	0.21	0.43	0.49
		$3 \cdot 10^{-5}$	10	air	nil	0.39	—
		$8 \cdot 10^{-5}$	10	air	0.26	0.33	0.78
		$2.8 \cdot 10^{-4}$	10	air	0.29	0.40	0.73

phosphorylations by *Ascaris* muscle preparations require, in addition, a non-dialyzable constituent of the parasite's perienteric fluid. Aerobic phosphorylations cannot be generated by the succinoxidase system of *Ascaris* muscle[11], because succinate failed to support the uptake of inorganic phosphate; furthermore, oxidation of this substrate inhibited aerobic phosphorylations.

Low concentrations ($3 \cdot 10^{-5}$ *M*) of dinitrophenol had the same effect on the metabolism of *Ascaris* muscle as on that of mammalian tissues, *i.e.*, they inhibited oxidative phosphorylations without reducing the oxygen uptake. However, if the concentration of dinitrophenol was raised about three times, uptake of inorganic phosphate consistently was greater than in the presence of the lower concentration and in some experiments it was equal to or even higher than that observed in the absence of dinitrophenol. It would appear that in *Ascaris* muscle the well-known "uncoupling" action of dinitrophenol is counterbalanced by a stimulatory effect of this compound on some mechanism concerned directly or indirectly in the aerobic utilization of inorganic phosphate.

Compared with mammalian tissues aerobic phosphorylations proceed at a much

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slower rate in *Ascaris* muscle. It remains to be determined whether this process is essential for this parasite or whether it represents merely a rudimentary function carried over from a previous stage of the parasite's life cycle or from a different, phylogenetically related species.

SUMMARY

The occurrence of aerobic phosphorylations in preparations of *Ascaris* muscle has been demonstrated. A particulate fraction of the muscle, a non-dialyzable component of the parasite's perienteric fluid and the presence of a substrate (pyruvate) and of diphosphopyridine nucleotide are required. In low concentrations dinitrophenol uncouples oxidative phosphorylations, but this effect is less pronounced or even abolished in the presence of higher concentrations of dinitrophenol.

RÉSUMÉ

Les auteurs ont démontré que des préparations de muscle d'*Ascaris* sont le siège de phosphorylations aérobies. Il faut pour cela qu'une fraction figurée du muscle, un composé non dialysable du fluide périentérique du parasite, un substrat (pyruvate) et du diphosphopyridine nucléotide soient présents. A faible concentration, le dinitrophénol dissocie les phosphorylations oxydatives, mais cet effet est moins prononcé ou même supprimé si la concentration en dinitrophénol est plus élevée.

ZUSAMMENFASSUNG

Das Vorkommen aerobischer Phosphorylierungen in *Ascaris*-Muskelpräparaten wurde gezeigt. Es sind eine besondere Fraktion des Muskels, ein nicht dialysierbarer Bestandteil der perienterischen Flüssigkeit des Parasiten, die Gegenwart eines Substrats (brenztraubensaures Salz) und von Diphosphoridinnucleotid erforderlich. In niedrigen Konzentrationen löst Dinitrophenol oxydative Phosphorylierungen aus; dieser Effekt ist aber weniger deutlich oder verschwindet sogar bei höheren Konzentrationen von Dinitrophenol.

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