BBA 45772

ELECTRON TRANSPORT AND PHOSPHOLIPASE ACTIVITY IN ENCYSTING CELLS OF THE CILIATE *COLPODA STEINII*

J. TIBBS AND B. J. MARSHALL
Department of Biochemistry, The University of Dundee (Great Britain)
(Received August 12th, 1968)

SUMMARY

1. Ciliates and cysts of *Colpoda steinii* are rich in phospholipase activity and this interferes with the assay of the phospholipid-dependent parts of the electron-transport chain.

2. Succinate:phenazine methosulphate oxidoreductase decreases by about 30% when cells encyst indicating no major degradation of mitochondria.

3. This is supported by the speed with which cell respiration is restored on addition of hay infusion to cysts and by electron microscope studies already published.

INTRODUCTION

As part of the process of encystment, the ciliate *Colpoda steinii* surrounds itself by a thick protein wall rich in glutamic acid[1]. This process takes place in the absence of exogenous metabolites and the cell itself must supply the necessary components from intracellular material not essential to the mature cyst. Since the dormant cyst will not require the mitochondrial complement of the motile ciliate, mitochondria may become reduced in number or modified in structure. Such appears to be the case in *Hartmanella castellani*[2] where these organelles lose their respiratory activities and internal organisation. This paper describes some observations made on electron transport in Colpoda and some of the difficulties which arose consequent on the presence of an active phospholipase.

EXPERIMENTAL

Materials

Phenazine methosulphate and cytochrome *c* (isolated from horse heart and 90–100% pure based on a molecular weight of 12 270) were obtained from the Sigma Chemical. Lecithin was from egg yolk prepared and purified as described by PANGBORN[3]. Other chemicals were of "Analar" grade.

Unless otherwise stated, the cysts used were cells encysted for the first time from the culture in which they had grown[1] and were harvested 10 days after encystment. They were washed by centrifugation. Ciliates used were obtained from cysts by treating with hay infusion for 1.5–2 h at 25° and were concentrated by centrifugation at 150 × *g* for 2 min. They were resuspended, debris removed by several

centrifugations at 50 × *g* for 1 min and the free-swimming cells left behind in the supernatant washed several times with distilled water using centrifugation at 500 × *g* for 2 min each time.

Preparation of homogenates

Homogenates of cysts, ciliates and mouse kidney were made in water using a Potter–Elvejhem type of homogeniser with a teflon pestle (10 min for Colpoda, 30 sec for kidney). During and after homogenisation, the preparations were maintained at 0°. Counts of intact cells remaining were made using a haemocytometer and indicated that 50–70 % of the cysts and 100 % of the ciliates had been disrupted. In a few experiments, indicated in RESULTS, cysts and ciliates were disrupted by shaking with 1-mm diameter glass beads in a Mickle high-speed shaker for 10 min. For cysts this was more efficient than the Potter homogeniser but was not generally used owing to the possibility of enzyme inactivation.

Enzyme assays

The manometric assay of succinoxidase, based on that described by POTTER[4], was carried out at 30°, each Warburg flask containing in the main compartment 80 μmoles of phosphate buffer (pH 7.4), 0.02 μmoles of cytochrome *c*, 0.8 μmoles of $CaCl_2$ and 0.8 μmoles of $AlCl_3$. The side arm contained 100 μmoles of succinate (pH 7.4) and the centre well KOH. Homogenates and water were included in the main compartment to give a final volume of liquid after mixing of 3.0 ml.

Succinate dehydrogenase (succinate: phenazine methosulphate oxidoreductase, EC 1.3.99.1) was assayed by the method of SINGER AND KEARNEY[5]. The main compartment of the Warburg flask contained 150 μmoles of phosphate (as buffer, pH 7.6) and 3.0 μmoles of cyanide (prepared immediately before use by adding an equal volume of 0.01 M HCl to 0.01 M NaCN). The side arm contained 60 μmoles of succinate (as the sodium salt, pH 7.6), and 2 mg of phenazine methosulphate. The latter was stored in the dark as a 1 % (w/v) frozen solution and 2 mg dye per flask gave the maximum rate of reaction. Homogenate and water were included in the main compartment to give a final volume of liquid after mixing of 3.0 ml.

For assaying[4] cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) the main compartment of each Warburg flask contained 80 μmoles of phosphate (as buffer, pH 7.4), 0.8 μmoles of $CaCl_2$, 0.8 μmoles of $AlCl_3$ and 40 μmoles of ascorbate (prepared immediately before use by neutralisation of the free acid with 0.1 M NaOH). The side arm contained 0.2 μmoles of cytochrome *c* and the centre well KOH. Homogenate and water were included in the main compartment to give a final volume of liquid after mixing of 3.0 ml.

Succinate: cytochrome *c* oxidoreductase was measured by following the change in absorbance of a solution of cytochrome *c* at 550 nm and 20° (ref. 6). The cuvettes contained in a final volume of 3.0 ml, 186 μmoles of phosphate (as buffer, pH 7.6), 5 μmoles of cyanide (as used for the succinate dehydrogenase assay), 260 μmoles of succinate (as the sodium salt, pH 7.6) and homogenate. The reaction was started by adding approx. 0.1 μmoles of cytochrome *c*. The results were calculated assuming a molar extinction coefficient difference of $1.97 \cdot 10^4$ between the oxidised and reduced forms of cytochrome *c*[6]. The amount of added cytochrome *c* was such that its total reduction by dithionite gave an absorbance change of 0.46.

Phospholipase activity was assayed under N_2 at 30° and pH 7.5 using a Radiometer (Copenhagen) automatic titrator as a pH-stat. The cup contained, in a final volume of 3 ml, homogenate and 25 μmoles of lecithin (calculated on the basis of the phosphorus content and dispersed as an emulsion in water by treating at 0° for 20 min with a 13 kcycles ultrasonic oscillator). 10 μmoles of $CaCl_2$ were added to activate the phospholipase[7,8]. Acid released due to the spontaneous breakdown of the lecithin and acid released in the absence of added lecithin were taken as controls.

RESULTS

Respiration measurements on intact cells

The respiration rates for two preparations of *C. steinii* cells during encystment and excystment are shown in Fig. 1. Table I summarises the Q_{O_2} (cell), Q_{CO_2} (cell) and the respiratory quotients obtained. The state of the cells was easily followed

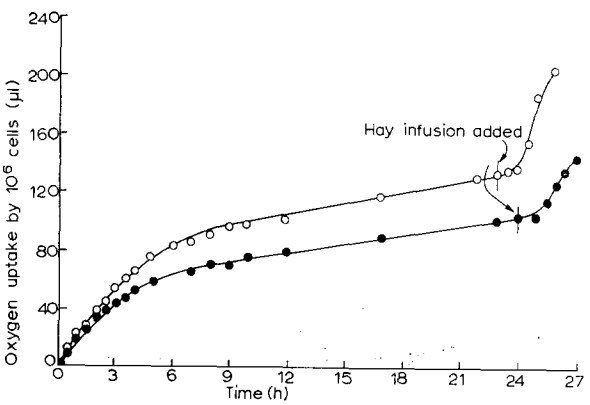

Fig. 1. O_2 uptake at 30° by two preparations of *C. steinii* cells during encystment and excystment. Purified ciliates in water (1 ml) were allowed to encyst in Warburg flasks which contained 0.2 ml of hay infusion in the side arms. Excystment was induced by tipping at the times indicated. The state of the cells throughout was followed microscopically by sampling from other flasks from which CO_2 had not been excluded and also macroscopically (see text). In both cases the cells showed a high degree of synchrony. At 1.5 h they had become immobile prior to encystment; at 6 h the cyst coat was partially formed and at 9 h it appeared complete but the cell cytoplasm could be observed moving. At 18 h no cytoplasmic movement could be detected but 1.5 h after the addition of hay infusion the cells had completely excysted.

TABLE I

O_2 AND CO_2 EXCHANGE IN *C. steinii* CELLS

Details as in Fig. 1. Q_{CO_2} values were measured by the Warburg direct method. CO_2 retention was ignored since the unbuffered pH of the suspensions, with and without hay infusion, was about 5.5.

	Starved ciliates prior to encystment		*Encysted cells*		*Excysted cells*	
	Prep. 1	*Prep. 2*	*Prep. 1*	*Prep. 2*	*Prep. 1*	*Prep. 2*
$10^6 \times Q_{O_2}$ (cell)	18.5	17.0	1.8	2.5	22.0	30.0
$10^6 \times Q_{CO_2}$ (cell)	11.1	11.5	0.7	1.4	12.5	23.7
Respiratory quotient	0.6	0.7	0.4	0.6	0.6	0.8

macroscopically as well as microscopically for on encystment the turbid suspension clears and clumps of cells are formed, many of which adhere to the flask and give it an etched appearance. Cells in the absence of CO_2 did not appear to behave differently from those in its presence. Respiratory quotients are low and the results show the decline in respiration which takes place when the cell encysts. They also indicate the speed with which normal respiration is resumed after the addition of hay infusion. This point was shown more clearly when a more concentrated suspension of cysts, harvested from the culture in which they had grown and exhibiting a Q_{O_2} (cell) of $6.3 \cdot 10^{-6}$ were treated with hay infusion. The response was immediately detectable and within 30 min the cells, which did not excyst until after 1 h had elapsed, were respiring with a Q_{O_2} (cell) of $28.2 \cdot 10^{-6}$. The value for the fully excysted cells was $39.4 \cdot 10^{-6}$.

Succinoxidase and cytochrome oxidase assays

In eight measurements of succinoxidase activity neither cyst nor ciliate homogenates had Q_{O_2} (N) values greater than 20, this corresponding to the limits of sensitivity of the assay. Under the same conditions mouse kidney homogenates gave Q_{O_2} (N) values of 600 to 750. The negative results with cysts could have been the result of mitochondrial degradation on encystment but, when mouse-kidney homogenates were mixed with approximately equal amounts, on a nitrogen basis, of cyst or ciliate homogenate and incubated together for 1 h at 30°, the succinoxidase activity was abolished. Mouse kidney homogenates alone, under these conditions of incubation, retained their succinoxidase activity.

When cytochrome oxidase was assayed, three ciliate preparations had Q_{O_2} (N) values of less than 20 whilst three cyst homogenates had Q_{O_2} (N) of 130–170. When cyst or ciliate homogenates were mixed with an approximately equal amount of mouse kidney homogenate nitrogen and incubated together for 1 h at 30°, the cytochrome oxidase activity of the latter was reduced by 50%–70% from values of around 1500. Thus, both cyst and ciliate homogenates inhibit the cytochrome oxidase activity of mouse kidney homogenates but perhaps not so efficiently as they inhibit the succinoxidase activity.

Nature and specificity of inhibitor

EICHEL[9,10] found difficulty in measuring electron transport in homogenates of *Tetrahymena pyriformis* and concluded that this was due to the presence of a phospholipase A. Experiments with mixtures of mouse kidney and Colpoda homogenates showed that the inhibition of the system assayed was higher when the mixture was allowed to stand before measuring O_2 uptake. Such an observation is in accordance with an inhibition resulting from enzymatic action and as standard procedure in assays involving inhibition, all mixtures of homogenates and controls were incubated for 1 h at 30° before measurement.

Cysts and ciliates were homogenised in 0.1 M phosphate buffer (pH 7.5) and aged for 4 h at 30°. The homogenates were exhaustively dialysed and the diffusate and non-diffusible fractions freeze-dried and made up to the original homogenate volume. Even after heating for 30 min at 100°, the diffusate inhibited mouse-kidney homogenate succinoxidase completely. The non-diffusible fraction also inhibited mouse-kidney succinoxidase 100% but this was reduced to approx. 30 and 20% after heating it at 100° for 5 and 30 min before preincubation of the mixture. These results suggest that

inhibition by the complete homogenate is partially due to a non-diffusible and fairly heat-stable substance and partially due to a heat-stable small molecule. These substances could be a phospholipase and the products of phospholipase action. Phospholipases are known to be remarkably heat-stable[11]. This experiment was repeated twice.

The effects of the inhibitor on the constituents of the succinoxidase chain were measured. Both cyst and ciliate homogenates exhibited succinate dehydrogenase activity (see later) and, when cyst or ciliate homogenates were mixed with approximately equal amounts of mouse kidney homogenate nitrogen, no inhibition of the enzyme was observed, the O_2 uptakes observed being additive.

After incubating mouse kidney and Colpoda homogenates (normal strength diluted 10 times with phosphate buffer (pH 7.6)), both separately and when mixed, for 1 h at 30°, 0.1-ml samples were assayed for succinate: cytochrome *c* oxidoreductase activity. The enzyme system could not be detected in either cyst or ciliate homogenates. In the mixed systems the activity of the mouse kidney homogenates was inhibited 100%.

T. pyriformis, strain W, was grown for 5 days at 30° on a solution of 0.2 % Difco bactotryptone and 0.2 % Difco yeast extract and harvested by centrifugation at 150 × *g* for 90 sec. After washing, the cells were homogenised and, together with homogenates of *C. steinii* ciliates and cysts were assayed for phospholipase activity (Table II). *C. steinii* cells in both forms hydrolysed phospholipid a good deal more efficiently than *T. pyriformis*.

TABLE II

PHOSPHOLIPASE ACTIVITY OF HOMOGENATES

Method of assay described in text

Homogenate	*Acid released from endogenous substrate* (*μmoles H^+/min/mg N*)	*Acid released from added lecithin* (*μmoles H^+/min/mg N*)
T. pyriformis	0.009	<0.005
C. steinii ciliates	0.057	0.091
	0.027	0.053
	0.028	0.030
C. steinii cysts	0.038	0.057
	0.038	0.025
	0.037	0.016

Since Ca^{2+} activate phospholipase[7,8] the effect of adding a specific calcium chelating agent to the homogenisation medium was tried. There was no increase in either succinoxidase or cytochrome oxidase activity when *C. steinii* cells (cysts and ciliates) were disrupted in 10 mM ethyleneglycol-bis-(β-aminoethyl ether *N-N'*-tetraacetic acid) containing 20 mM $MgCl_2$.

Possible effects due to contaminating bacteria

When concentrated suspensions of *C. cloacae* (the organism on which the protozoa were fed) were homogenised in the same way as *C. steinii*, mixed with equal

amounts of mouse-kidney homogenate nitrogen and incubated at 30° for 1 h, they had no effect on the succinoxidase of the mouse kidney. Neither did they have any detectable phospholipase activity. These concentrations of bacteria were far in excess of those which could have contaminated the protozoan preparations.

Succinate dehydrogenase activity in cysts and ciliates

Succinate dehydrogenase, the only enzyme measured which was unaffected by the presence of the inhibitor, was assayed in cysts from culture and in ciliates obtained from such cysts. Q_{O_2} (N) values of 150 and 210 were obtained from two preparations of cysts and values of 156 and 159 for two preparations of ciliates.

The enzyme was also measured in encysting cells, these being broken by a Mickle shaker rather than the more usual Potter homogeniser. Three preprarations of ciliates had Q_{O_2} (cell) values of $6.4 \cdot 10^{-6}$, $7.5 \cdot 10^{-6}$ and $7.6 \cdot 10^{-6}$, whilst the cysts from these ciliates had Q_{O_2} (cell) values of $4.6 \cdot 10^{-6}$, $5.3 \cdot 10^{-6}$ and $4.9 \cdot 10^{-6}$ respectively. The cysts therefore had 64–72 % of the ciliate activity.

In a separate experiment in which the enzyme was measured as a function of the state of the cells during encystment, it was found that this reduction in activity occurred during the first 3 h after settling.

DISCUSSION

Homogenates of *C. steinii* possess phospholipase activity. This would account for the lack of succinoxidase activity in such preparations and explain the inhibition patterns observed with mouse kidney homogenates. Phospholipid is probably an essential component of the succinoxidase chain[12] and is also involved structurally[13]. Succinate dehydrogenase with phenazine methosulphate as acceptor does not require phospholipid and is not, in fact, inhibited by Colpoda homogenates. It is also readily detectable in such homogenates. Both succinate cytochrome *c* reductase and cytochrome oxidase need phospholipid, the former more than the latter [14,15]. Thus, succinate cytochrome *c* reductase is completely inhibited by Colpoda homogenates whereas cytochrome oxidase is only inhibited about 40 % under the same conditions. FLEISCHER, CASU AND FLEISCHER[16] found that inhibition of mammalian electron transport by phospholipase A was produced partly by fatty acids released and partly by structural changes. Inhibition of mouse kidney succinoxidase activity by *C. steinii* homogenates is partly due to a heat-stable diffusible molecule and partly to a fairly heat-stable large molecule. EICHEL[9,10] published similar results using various strains of *T. pyriformis* and concluded that the inhibitor in that organism was phospholipase A. There appears to be more phospholipase activity in *C. steinii* than in *T. pyriformis* (strain W) but there is no reason to suggest that it is connected directly with encystment.

Succinate dehydrogenase activity decreases only by about 30 % during encystment and this suggests that no substantial degradation of mitochondria occurs. This is supported by the speed with which cysts exhibit a normal ciliate respiration rate on treating with hay infusion. 30 min seems too short a period for the synthesis of significant amounts of mitochondrial enzymes. TIBBS[17], studying electron micrographs of cell sections, found neither a reduced number of mitochondria in cysts nor any loss of intramitochondrial organisation. Possibly as the result of a reduction in cell volume the tubular system of the mitochondria was more regular and close-packed in the

encysted cell. It is of interest to compare these results with those obtained with *Hartmanella castellani*[2]. The mitochondria of this amoeba lose their oxidative and phosphorylating ability and also their internal organisation on encystment.

The exclusion of CO_2 from excysting cells did not delay or inhibit excystment. CO_2 has been suggested as an excystment trigger in *Naegleria gruberi*[18] but apparently plays no such role in *C. steinii*.

REFERENCES

1 J. TIBBS, *Biochem. J.*, 98 (1966) 645.
2 A. J. GRIFFITHS, D. LLOYD, G. I. ROACH AND D. E. HUGHES, *Biochem. J.*, 103 (1967) 21 P.
3 M. C. PANGBORN, *J. Biol. Chem.*, 188 (1951) 471.
4 V. R. POTTER, in W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques*, Burgess, Minneapolis, 4th Ed., 1964, p. 162.
5 T. P. SINGER AND E. B. KEARNEY, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 4, Interscience, New York, 1957, p. 307.
6 D. E. GREEN, S. MII AND P. M. KOHOUT, *J. Biol. Chem.*, 217 (1955) 553.
7 S. W. EDWARDS AND E. G. BALL, *J. Biol. Chem.*, 209 (1954) 619.
8 C. LONG AND I. F. PENNY, *Biochem. J.*, 65 (1957) 382.
9 H. J. EICHEL, *Biochim. Biophys. Acta*, 34 (1959) 589.
10 H. J. EICHEL, *J. Protozool.*, 7 (1960) 19.
11 O. HAYAISHI, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 660.
12 Y. H. ABDULLA AND A. N. DAVISON, *Biochem. J.*, 96 (1965) 10 C.
13 D. E. GREEN AND S. FLEISCHER, in R. M. C. DAWSON AND D. N. RHODES, *Metabolism and Physiological Significance of Lipids*, Wiley, London, 1964, p. 581.
14 A. P. NYGAARD AND J. B. SUMNER, *J. Biol. Chem.*, 200 (1953) 723.
15 S. W. EDWARDS AND E. G. BALL, *J. Biol. Chem.*, 209 (1954) 619.
16 S. FLEISCHER, A. CASU AND B. FLEISCHER, *Federation Proc.*, 23 (1964) 486.
17 J. TIBBS, *J. Protozool.*, in the press.
18 M. AVERNER AND C. FULTON, *J. Gen. Microbiol.*, 42 (1966) 245.