OXIDATIVE PHOSPHORYLATION IN CRITHIDIA FASCICULATA

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Extensive research has been conducted in respiratory chain-linked phosphorylation with preparations from mammalian tissues, plants, bacteria, and yeast, but little work in this area has been reported with protozoa (Eichel and Rem, 1961; Nishi and Scherbaum, 1962; Buetow and Buchanan, 1965). Indeed, there has been some question as to the nature of the energy producing reactions in this group of microorganisms since many reports (Baernstein, 1963; Perini et al., 1964) indicate that, in comparison with mammalian mitochondria, certain components of the electron transport chain are either absent or are present in limiting amounts. It is of particular interest therefore to study the respiratory systems of such organisms.

In view of this, as part of a comparative study of terminal electron pathways in microbial systems, Crithidia fasciculata was chosen as the protozoan prototype since it contains coenzyme Q_9 (Kusel and Weber, 1965), cytochrome b, and cytochrome oxidase, and is apparently devoid of cytochrome c (Kusel and Weber, in preparation). This organism is especially attractive as an experimental system since abundant axenic growth can be obtained in vitro (Cowperthwaite et al., 1953), and since it contains a long mitochondrial-type organelle (Kusel et al., 1967), which, by analogy with mammalian systems, should contain the respiratory enzymes. This paper presents evidence for the coupling of phosphorylation to electron transport in cell-free extracts from

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this protozoan, using NADH and succinate as electron donors.

MATERIALS AND METHODS

Crithidia fasciculata, obtained from Dr. S. H. Hutner, was grown in a medium previously described (Kusel and Weber, 1965). The cells were grown to a density of 1.5 x 10^8/ml and harvested by centrifuging at 1600 x g for 10 minutes. This centrifugation and all subsequent procedures were carried out at 0-4° C. The cells were washed by suspending in 0.25 M sucrose contained in 0.02 M Tris-HCl buffer, pH 8.0 and centrifuging at 2000 x g for 7 minutes. The cells were resuspended in the sucrose-Tris buffer to a 20% wet weight suspension (w/v) and disrupted either by passage through a French pressure cell at 7500 psi, or by sonic vibration for 1 minute in a 10 kc Raytheon Sonic Oscillator tuned to maximum output at 1.25 amp. Comparable results were obtained by either method. The crude extracts were centrifuged at 300-800 x g to remove whole cells and debris, and the supernatant fluids were collected and assayed immediately.

Assays for oxygen consumption were performed manometrically at 30° C on the Gilson Differential Respirometer (Gilson Medical Electronics) using air as the gas phase. The reaction vessels were thermo-equilibrated by a 10 minute preincubation prior to tipping in substrate from the sidearms to start the reactions. Inorganic phosphate was assayed by the Fiske-SubbaRow (1925) procedure. Phosphate disappearance from the medium was used as a measure of phosphate esterification, and was determined by comparing each reaction vessel with an identical "zero time" control. Protein was estimated by the method of Lowry et al. (1951).

NADH, ATP, hexokinase, and the antibiotics were obtained from Sigma Chemical Company. Carbonyl cyanide m-chlorophenylhydrazone (mCl-CCP)

was obtained from Calbiochem and was added as an aqueous solution of the Tris salt.

RESULTS AND DISCUSSION

Results from typical experiments are presented in Tables I and II. It was necessary to use relatively large amounts of extract protein (14-30 mg) in the

TABLE I

OXIDATIVE PHOSPHORYLATION WITH NADH AS SUBSTRATE

Reaction mixture	Oxygen uptake μ atoms	Δ Pi μ moles	P/O
Experiment 1			
complete	3.42	2.49	0.73
-acceptor system	3.20	0	0
-substrate	0.98	0	0
+Antimycin A (1 μg/ml)	0.92	0	0
+KCN (1 μmole/ml)	0.64	0	0
Experiment 2			
complete	2.97	3.31	1.11
+gramicidin (6.7 μg/ml)	2.93	1.61	0.55
+ETOH	3.03	3.24	1.07
Experiment 3			
complete	3.99	2.80	0.70
+mCL-CCP (10^{-5} M)	3.08	0	0

Reaction vessels contained, in the main compartment, 0.2 mmoles of Tris-HCl buffer, pH 8.0, 0.5 mmoles of sucrose, 15 μmoles of KPO_4, 40 μmoles of KF, 3 μmoles of $MgCl_2$, 10 μmoles of ATP, 50 μmoles of glucose, 1 mg of hexokinase (Sigma type V), and 27.3 mg, 28.6 mg, 23.8 mg of extract protein in experiments 1, 2, and 3, respectively. Inhibitors, where used, were placed in the main compartment except for KCN which was added with substrate (10 μmoles of NADH) from the sidearm. 0.2 ml of 20% KOH was present in the center well, except for the KCN experiments when 0.2 ml of a balanced $Ca(CN)_2$-$Ca(OH)_2$ mixture (Robbie, 1948) was used. Final reaction volume was 2.5 ml. When the vessel containing the complete reaction mixture had reached 3-4 μ atoms of oxygen uptake (9-15 minutes), the reactions were terminated by immediate chilling in an ice bath and addition of 1.0 ml of cold 10% TCA.

reactions in order to obtain optimal P/O ratios. A hexose-hexokinase-ATP phosphate acceptor system was required, and ATP was an essential component of this acceptor system in order to obtain phosphorylation.

As found with other microbial systems, the P/O ratios obtained were lower than those observed with mammalian mitochondrial preparations. Data from a large number of experiments with NADH as electron donor gave P/O ratios in the range of 0.5-1.6, with an average of about 0.9. With given preparations, P/O's obtained with succinate as substrate were usually lower than with NADH, averaging about 0.6-0.7.

The Crithidia preparations were capable of coupling phosphorylation to respiration, but were not "tightly coupled". The oxygen consumption observed was relatively independent of a phosphate acceptor system (Experiment 1, Table I), although, interestingly, a slight depression in respiratory rate was frequently observed when either ATP alone or the entire phosphate acceptor system was omitted from the reaction mixture.

TABLE II

OXIDATIVE PHOSPHORYLATION WITH SUCCINATE AS SUBSTRATE

Reaction mixture	Oxygen uptake μ atoms	Δ Pi μ moles	P/O
complete	3.02	1.75	0.58
-substrate	0.48	0	0
+mCl-CCP (10^{-5} M)	3.14	0	0
+Antimycin A (1 μg/ml)	0.43	0	0
+ETOH	2.90	1.75	0.60

Reaction mixtures are the same as in Table I except 40 μmoles of K-succinate was used as electron donor and 14.9 mg of extract protein was added.

As seen in Experiment 1, Table I, and the data in Table II, there were

high levels of endogenous respiration in the extracts, with no concomitant phosphorylation. The phosphorylating efficiency of the substrate-dependent respiration may therefore have been greater than the P/O ratios indicate.

The results obtained with respiratory inhibitors and uncoupling agents were similar whether NADH (TableI) or succinate (Table II) was used as substrate. The substrate-dependent respiration was apparently blocked by the respiratory inhibitors KCN and Antimycin A since in the inhibited reactions oxygen uptake equaled the endogenous level. As expected, no phosphate uptake was observed in these reactions.

The effectiveness of gramicidin and mCl-CCP as uncoupling agents can be seen in Tables I and II. Although not indicated here, gramicidin was usually found to inhibit respiration to a slight extent. It inhibited phosphorylation to a greater degree, however, causing a decreased P/O. mCl-CCP showed either no effect or, occasionally, a slight depressive effect on respiratory rate. It was found to completely uncouple phosphorylation at the concentration indicated. Stimulation of the respiration rate was not observed with any of the uncoupling agents used.

Since the antibiotic agents were added as ethanolic solutions, reactions with equal amounts of ethanol were run as controls. No alterations of the P/O ratio were observed with the control reactions.

SUMMARY

Respiratory chain-linked phosphorylation has been demonstrated in cell-free extracts from the protozoan Crithidia fasciculata. The preparations were capable of coupling phosphate esterification to respiration, but were not "tightly coupled". Oxidation and phosphorylation were sensitive to respiratory inhibitors and uncoupling agents. The P/O ratios observed with NADH

and succinate as electron donors were lower than those obtained with mammalian mitochondria, but were typical for microbial systems.

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