While this work was in progress, Toner and Weber (7) working independently and using a different method, isolated a mitochondrial preparation from C. fasciculata which shows energy coupling. The results from both laboratories are in essential agreement.

MATERIALS AND METHODS

Crithidia fasciculata (A.T.C.C. No. 11745) was grown under the conditions previously described (8), with the following changes in the growth medium: hemin concentration was 3 μM; 1.5% triethanolamine HCl (Sigma) replaced the free base; and Tween 80 was omitted. Sterile hemin solution was added to the medium just prior to inoculation. Organisms grew with a doubling time of six hours to a stationary phase density of 2×10^8 cells/ml. Cells were collected with a Sharples continous-flow centrifuge.

Preparation of Mitochondria: Unwashed cells were resuspended to 5% w/v in ice-cold isolation medium containing 0.25 M mannitol, 10 mM morpholino-propane sulfonic acid (MOPS), 2.0 g/l polyvinylpyrrolidone (PVP), and 3.0 g/l defatted bovine serum albumin (BSA) (fraction V, Nutritional Biochemicals Corp.), 4.5 mM ascorbate, 250 μM $MgCl_2$ and 250 μM EDTA; the pH was adjusted to 8.0 with KOH. Digitonin in dimethylformamide (40 mg/ml) was added dropwise to the cell suspension with rapid stirring to provide 6.0 mg/g cells. After slow stirring for 30-40 minutes at 0-4°, the cells were recovered by centrifugation at 1,500 x g for 10 minutes, and were resuspended to 10% w/v in isolation medium. Cells were disrupted with the Polytron blendor (Bronwill Scientific Co.) using one 30 sec. treatment at maximum speed with addition of an equal volume of isolation medium after 20 sec.; lysis was 75-90%. Unbroken cells and debris were removed by centrifugation at 680 x g for 10 min. Centrifugation of the supernatant at 12,000 x g for 10 min. yielded a crude mitochondrial pellet. The latter was resuspended with a glass-teflon homogenizer in wash medium, using approximately 1 ml of wash medium per 5 ml of crude homogenate. Wash medium was identical with isolation medium but without ascorbate. Upon centrifugation at 12,000 x g for 10 min., a three-layered sediment was obtained. The supernatant and upper layer

EVIDENCE FOR THE PRESENCE OF TWO PHOSPHORYLATION SITES IN MITOCHONDRIA ISOLATED FROM THE TRYPANOSOMATID HEMOFLAGELLATE, CRITHIDIA FASCICULATA

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SUMMARY

Mitochondrial preparations have been isolated from Crithidia fasciculata which show respiratory control with ADP as phosphate acceptor with endogenous substrate, and with succinate or L-1-glycerolphosphate. Two energy coupling sites between succinate and oxygen are operative, corresponding to sites II and III in mitochondria isolated from other sources.

Particulate preparations have been isolated from Crithidia fasciculata which oxidize succinate and contain a full complement of cytochromes, differing somewhat in relative amounts and significantly in absorbance maxima from those of mitochondria isolated from animal and plant tissues (1,2,3). These preparations lacked respiratory control (1,4) and the ADP/O ratio with succinate as substrate, assayed with glucose plus hexokinase, was 0.5 to 1.0, suggesting that but one coupling site exists between succinic dehydrogenase and O_2 in this organism. Buetow and Buchanan (5) interpreted the low ADP/O ratios found in mitochondria from Euglena gracilis in this manner, but Sharpless and Butow (6) later demonstrated that the expected two sites were indeed operative in these mitochondria between succinate and O_2. We have now shown that this is also true of mitochondria isolated from C. fasciculata: the mitochondria isolated as reported in this paper have two energy coupling sites corresponding to sites II and III in mitochondria from higher organisms.

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were removed by aspiration. The brown mitochondrial layer and underlying viscous white button were resuspended in half the previous volume of wash medium. Particulate aggregates were removed by centrifugation at 480 x g for 5 min. The supernatant was then centrifuged at 12,000 x g for 10 min. The top layer of the pellet was carefully removed, and the brown mitochondrial layer was resuspended in one-fourth volume of wash fluid used in the first wash step, avoiding the viscous white underlayer. The last two centrifugation steps were repeated. The thrice-washed mitochondria were resuspended in a minimal volume of wash medium.

Oxidation rates and respiratory control ratios with ADP as Pi acceptor were determined as described by Estabrook (9) in a medium containing 0.25 M mannitol, 50 mM tris-(hydroxymethyl)-methylaminopropane sulfonic acid (TAPS), and 5 mM KP_i at pH 7.5. The concentration of ADP in the stock solution was determined enzymatically as described by Williamson and Corkey (10). Protein concentration of the final mitochondrial suspension was determined by a modified Lowry method (11). The uncouplers p-trifluoromethoxy-carbonylcyanide-phenyl-hydrazone (FCCP) and bis-(hexafluoroacetonyl)-acetone (1799) were kindly supplied by Dr. P. Heytler of E.I. duPont de Nemours Co. and were used as ethanolic solutions. N,N'-tetramethylpheylenediamine (TMPD) and $K_3Fe(CN)_6$ were recrystallized prior to use.

RESULTS AND DISCUSSION

The time course of O_2 uptake by the mitochondrial preparation from C. fasciculata, isolated as described above, is shown in Fig. 1. Respiratory control is observed with endogenous substrate (A), and with succinate and L-1-glycerophosphate as exogenous substrates (B and C). With succinate as substrate and ADP as P_i acceptor, the respiratory control ratio is 0.9-1.1. The uncoupler FCCP stimulated the rate of O_2 uptake to a greater extent than ADP with succinate (Fig. 1B) and with L-1-glycerolphosphate (not shown), but not with endogenous substrate (Fig. 1A). The respiratory control ratio calculated from the uncoupled

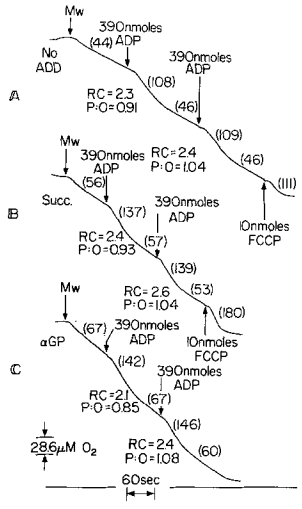

Figure 1. Mitochondrial protein (4.2 mg) was added to 3.8 ml of MTP medium supplemented with 2.5 mM $MgCl_2$ and 250 μM ATP, and either 6.6 mM potassium succinate (pH 7.5) or 13.2 mM DL-glycerol-1-phosphate where indicated. The numerals in parentheses indicate specific activities calculated as nanoatoms O $min.^{-1}$ mg $protein^{-1}$. Assay temperature was 22.5°. MTP: 0.25 M mannitol, 50 mM TAPS, 5 mM KPi, pH 7.5.

rate with succinate is 3.4.

The location of the energy coupling sites with respect to the respiratory chain carriers was determined with ascorbate plus TMPD in the presence of antimycin A to assay site III (12,13) and with the method of Sottocasa and Sandri (14) to assay site II plus site III. The time course of O_2 uptake with ascorbate plus TMPD is shown in Fig. 2. The rate is stimulated by the uncoupler 1799 (Fig. 2A); the ratio of the uncoupled to the state 4 rate is 1.4. A cycle of transition from state 4 to state 3 with ADP addition, followed by return to state 4 is shown in Fig. 2B. The respiratory control ratio is 1.2 and the ADP/O ratio is 0.3. The values for these ratios are low, but reproducible from preparation to preparation. The low values derive in part from the use of TMPD: at 135 μM, respiratory control is demonstrable, but the state 3 rate is not maximal. Higher concentrations give faster oxidation rates with complete loss of respiratory control, demonstrating a concentration-dependent uncoupling activity of TMPD.

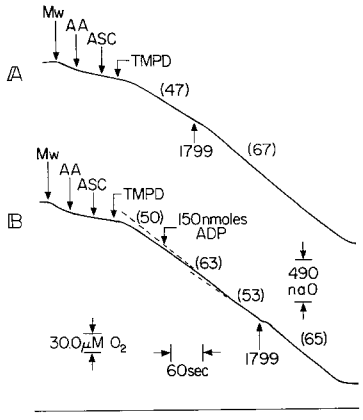

Figure 2. Mitochondrial protein (4.8 mg) was added to 3.7 ml of MTP medium supplemented with 2.5 mM $MgCl_2$. Subsequent additions were as follows: 0.5 μg/ml antimycin A; 6.7 mM ascorbate (pH 7.5); 135 μM TMPD. Assay temperature was 20.0°. Numerals in parentheses indicate specific activities as in Fig. 1.

Energy coupling at site II with succinate as substrate and $K_3Fe(CN)_6$ as acceptor is shown in Fig. 3. Addition of $K_3Fe(CN)_6$ decreases the amount of O_2 consumed during phosphorylation of added ADP; the decrease is proportional to the amount added (Figs. 3B and C). The ADP/2e ratio, calculated by the equations of Sottocasa and Sandri (14), is 0.36. The value of the ADP/2e ratio calculated by this method depends on the efficiency of $K_3Fe(CN)_6$ as acceptor of the reducing equivalents passing through site II with concomitant phosphorylation of ADP compared to that of O_2 as acceptor. The records of Sottocasa and Sandri (14) show a substantial decrease of the state 3 rate on adding $K_3Fe(CN)_6$ to respiring rat liver mitochondria, showing that $K_3Fe(CN)_6$ competes effectively with O_2 in this system. The relatively small decrease in the state 3 rate in respiring mitochondria from C. fasciculata (Figs. 3B and C) shows that $K_3Fe(CN)_6$ competes much less effectively with O_2, resulting in a low ADP/2e ratio.

The estimates of the ADP/2e ratio at site III with ascorbate plus TMPD with O_2 as acceptor, and at site II with succinate plus $K_3Fe(CN)_6$ as acceptor, both give lower limits for the reasons cited. Since the ADP/O ratios with

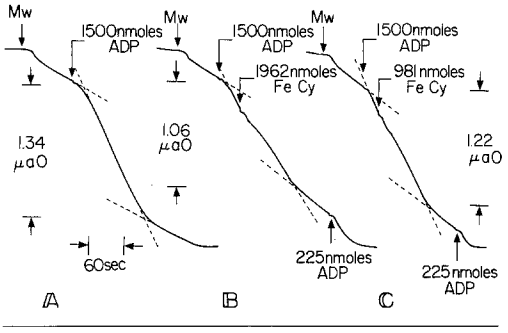

Figure 3. Mitochondrial protein (3.6 mg) was added to 3.7 ml of MTP medium supplemented with 2.5 mM $MgCl_2$ and 6.6 mM potassium succinate (pH 7.5). $K_3Fe(CN)_6$ was added at point marked "FeCy". Assay temperature was 22.0°.

succinate as substrate are consistently in the range 0.9 to 1.1 with these mitochondrial preparations, we conclude that both sites II and III are operative with ADP/2e ratios of 0.5 to 0.6 at each site. The reasons for the low efficiency of phosphorylation are not clear at present. It may be due in part to the presence of cytochrome o (1) and/or a microsomal oxidase as contaminant (3), which consume O_2 without phosphorylation of ADP. Work is currently underway to check this hypothesis. As has been pointed out, however, mitochondria isolated from Euglena gracilis also have two coupling sites, yet exhibit low phosphorylation efficiency (5,6). It may be that this property is inherent to the lower orders of unicellular eukaryotes. Demonstration of this requires further investigation of mitochondria from this trypanosomatid. Such work is now in progress.

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REFERENCES

1. Hill, G. C. and White, D. C., J. Bacteriol. 95: 2151 (1968).
2. Kusel, J. P. and Weber, M. M., Arch. Biochem. Biophys. 133: 293 (1969).
3. Kusel, J. P., Ph.D. Dissertation, St. Louis University, 1970.
4. Toner, J. J. and Weber, M. M., Biochem. Biophys. Res. Comm. 28: 821 (1967).
5. Buetow, D. E. and Buchanan, P. J., Biochim. Biophys. Acta 96: 9 (1965).
6. Sharpless, T. K. and Butow, R. A., J. Biol. Chem. 245: 50 (1970).
7. Toner, J. J. and Weber, M. M., Biochem. Biophys. Res. Comm., 000 (1971).
8. Kusel, J. P. and Weber, M. M., Biochim. Biophys. Acta 98: 632 (1965).
9. Estabrook, R. W., in Methods in Enzymology, Vol. X (R. W. Estabrook and M. E. Pullman, eds.), Academic Press, New York, 1967, p. 41.
10. Williamson, J. R. and Corkey, B. E., in Methods in Enzymology, Vol. XIII (J. M. Lowenstein, ed.), Academic Press, New York, 1969, p. 434.
11. Miller, G. L., Anal. Chem. 31: 964 (1954).
12. Sanadi, D. R. and Jacobs, E. E., in Methods in Enzymology, Vol. X (R. W. Estabrook and M. E. Pullman, eds.), Academic Press, New York, 1967, p. 38.
13. Tyler, D. D., Estabrook, R. W. and Sanadi, D. R., Arch. Biochem. Biophys. 114: 239 (1966).
14. Sottocasa, G. L. and Sandri, G., Biochim. Biophys. Acta 223: 36 (1970).