EVIDENCE FOR P/O RATIOS APPROACHING 6 IN MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

Archie L. Smith and Marc Hansen
with the technical assistance of Sandra Rolland

Institute for Enzyme Research and Department of Pediatrics
University of Wisconsin, Madison, Wisconsin

Received March 20, 1964

Mitochondria couple the synthesis of ATP to oxidative reactions of the electron transfer chain, and the understanding of this coupling has been a major biochemical objective. The efficiency of ATP synthesis has been reported in terms of phosphate esterified per oxygen consumed (P/O ratios). Ratios of 3 for pyruvate, malate, glutamate, and 2 for succinate have been observed in mitochondria from a wide variety of tissues and species. These ratios have been considered to be the highest that can be achieved and have thus been considered to be the "theoretical" P/O ratios.

Hatefi and Lester (1958) and Hatefi et al. (1961) examined oxidative phosphorylation in beef heart mitochondria and found phosphorylation efficiencies similar to those described by other workers. We have re-examined the question of phosphorylation efficiency in beef heart mitochondria and submitochondrial particles. P/O ratios as high as 5.7 have been observed, and these high P/O ratios are dependent on the integrity of the preparation.

Methods: Oxidative phosphorylation was measured by conventional manometric techniques at 30° as described previously (Hansen and Smith, 1964). After a five minute thermal equilibration period in the presence of substrate and phosphate the reaction was initiated by the addition of ADP and hexokinase.

Results: In our studies of phosphorylation in ETP_H (Hansen and Smith, 1964) it was noted that assay conditions greatly influenced the P/O ratio. We had shown that the highest P/O values were obtained by allowing the particle to oxidize substrate in the absence of ADP during the thermal equilibration period and

then initiating synthesis of ATP by the addition of ADP and hexokinase. This technique was employed to study oxidative phosphorylation in intact heavy beef heart mitochondria (HBHM). As examples, P/O ratios of 4.4, 5.0, and 5.7 have been obtained with pyruvate as substrate with three different preparations of HBHM (see Table 1) that were prepared by treatment of heart muscle with the proteolytic enzyme, Nagarse (Hatefi et al., 1961; Chance and Hagihara, 1961). These high P/O ratios have been obtained in every preparation of HBHM that has been examined. Similarly, P/O ratios of 2.7 and 3.9 have been obtained with succinate as substrate. Although not shown in the Table, P/O ratios of from 4 - 5 have been observed with glutamate as substrate. When mitochondria are prepared by the large scale procedure of Crane et al. (1956) and the HBHM isolated according to Hatefi and Lester (1958), the P/O ratio with pyruvate as substrate is about 4.0.[a] In these preparations the P/O ratio with succinate has never exceeded 2.0. When the substrate was omitted during the assay there was no disappearance of inorganic phosphate throughout the incubation period. The phosphorylation in all the experiments reported is inhibited by dinitrophenol (DNP) - an observation that excludes the possibility that the "extra" phosphorylation is substrate-level linked.

These observations of high P/O ratios were extended to the submitochondrial particle ETP_H. We have previously reported that the highest P/O values for ETP_H were obtained at low oxidation rates (Hansen and Smith, 1964). The oxidation rate of ETP_H can be regulated by adjusting the DPNH generating system (alcohol dehydrogenase and DPN). As shown in Table 2, when the DPNH oxidation rate was reduced to approximately 0.1 μatoms O_2/min/mg protein, P/O ratios of 4 or greater were observed. The high phosphorylation ratios with ETP_H are also sensitive to the uncoupling agent DNP.

---

a Note: The observation of P/O ratios in excess of 3.0 has been observed in 20 out of 21 different preparations of HBHM and 6 out of 9 ETP_H preparations. These observations of high P/O ratios have been confirmed in two other laboratories at this Institute (Dr. David MacLennan and Dr. Sidney Fleischer).

TABLE 1

Oxidative Phosphorylation of HBHM

Enzyme	Preparation Number	Substrate	ΔO_2 (µatoms)	S.A.[3]	ΔP_i (µmoles)	P/O
HBHM[1]	1	Pyruvate	6.2	0.22	27.2	4.4
	2	Pyruvate	5.7	0.20	28.6	5.0
	3	Pyruvate	5.5	0.20	31.3	5.7
	2	Pyruvate + DNP (10^{-4} M)	6.0	0.08	0.0	0.0
	2	Succinate	3.2	0.04	8.5	2.7
	3	Succinate	4.2	0.05	16.5	3.9
HBHM[2]	1	Pyruvate	5.2	0.19	20.9	4.0
	1	Pyruvate	5.9	0.21	22.2	3.8
	1	Succinate	4.8	0.12	8.2	1.7
	1	Succinate	4.2	0.11	6.8	1.6

The reaction mixture contained in 3.0 ml: 35 µmoles KPO_4 of pH 7.4, 15 µmoles $MgCl_2$, 100 µmoles glucose, 750 µmoles sucrose, 3.3 µmoles of K pyruvate and K malate or 25 µmoles K succinate, and 4.0 mg HBHM. Five µmoles ADP and 100 µg crystalline hexokinase (Worthington, 430 units/mg) were placed in the side arm of the reaction vessel.

1 HBHM prepared with Nagarse protease procedure of Hatefi et al. (1961).
2 HBHM prepared as described by Hatefi and Lester (1958).
3 S.A. µatoms O_2 uptake/min/mg protein.

TABLE 2

DPNH Linked Oxidative Phosphorylation in ETP_H

Enzyme Preparations	ΔO_2 S.A.[1]	ΔP_i S.A.[1]	P/O
1	0.08	0.36	4.5
2	0.11	0.42	3.7
3	0.10	0.39	3.9
4	0.09	0.37	4.0
4 + DNP (10^{-4} M)	0.13	0.04	0.3

The reaction conditions were the same as in the legend for Table 1 with the following exceptions: DPNH was generated with 500 µg alcohol dehydrogenase, 0.5 µmoles DPN, 10 µmoles ethanol, and 15 µmoles semicarbazide. One mg of ETP_H was used in the assay.

1 S.A. = µatoms O_2 or µmole P_i/min/mg protein.

Discussion: The observation of P/O ratios approaching 6 is dependent on several sets of conditions. Firstly, the methods of preparation of the HBHM and ETP_H are highly critical. The mitochondria prepared from beef heart mince exposed to the proteolytic enzyme Nagarse, consistantly exhibit the highest observed phosphorylation efficiency. At this time, we cannot specify the upper limit for the P/O ratio. Lower P/O ratios have been obtained when the HBHM are prepared according to Hatefi and Lester (1958). This method of preparation apparently yields particles which are partially damaged. The high P/O ratios have only been seen in ETP_H which have been prepared in the presence of Mg, Mn, ATP, and succinate (Hansen and Smith, 1964).

High P/O ratios were usually observed when the mitochondria or ETP_H are assayed immediately following preparation. When the preparations were allowed to stand at 0° for varying periods of time the phosphorylation efficiency declines. The aging phenomenon is most noticeable in ETP_H and has been pointed out previously (Hansen and Smith, 1964).

Another requirement for obtaining P/O ratios in excess of the "theoretical" value of 3 lies in the conditions which precede the actual measurement. If one allows the particles to oxidize substrate in the absence of the hexokinase trapping system (ADP and hexokinase) during the thermal equilibration period (5 min.), P/O ratios in excess of the "theoretical" of 3 for DPNH and 2.0 for succinate can be readily observed in both intact mitochondria and the sub-mitochondrial particle, ETP_H. The incubation of HBHM and ETP_H in the presence of substrate, magnesium, and phosphate would appear to lead to restoration of the phosphorylation mechanism. This "repair" may be via the generation of various high energy intermediates. The nature of the "repair" process is not known but has been observed in the restoration of ability of aged mitochondria to accumulate Ca ions (Brierley et al., 1964).

The observation of increased phosphorylation efficiency can be interpreted in several ways. The passage of 1 electron through each of 3 electron transfer complexes (Green et al., 1963) could lead to the formation of 3 ATP molecules. Since the oxidation of citric cycle substrates is a 2 electron process, one

might expect to observe P/O ratios of 6. Boyer (1963) recently pointed out that a phosphorylation mechanism of this type might be possible although no experimental proof was provided. Another interpretation of these findings is that there are more than three phosphorylation sites in the electron transfer system. Each of the additional phosphorylation sites might have coupling factors similar to those previously described (Green et al., 1963). Further studies are in progress to determine which of these two possibilities is more valid.

This work was supported in part by National Heart Institute Research Grant HE-00458 and National Institute of Health General Research Support Grant to the University of Wisconsin. Meat by-products were generously furnished by Oscar Mayer and Co., Madison.

Boyer, P. D., Science, 141, 1147 (1963).
Brierley, G. P., Murer, E., and Bachmann, E., Arch. Biochem. Biophys. (in press).
Chance, B. and Hagihara, B., Proc. 5th International Cong. Biochem., V, 3 (1961).
Crane, F. L., Glenn, J., and Green, D. E., Biochim. et Biophys. Acta, 22, 475 (1956).
Green, D. E., Beyer, R. E., Hansen, M., Smith, A. L., and Webster, G., Fed. Proceedings, 22, 1460 (1963).
Hansen, M. and Smith, A. L., Biochim. et Biophys. Acta, 81, 214 (1964).
Hatefi, Y., Jurtshuk, P., and Haavik, A. G., Arch. Biochem. Biophys., 94, 148 (1961).
Hatefi, Y. and Lester, R. L., Biochim. et Biophys. Acta, 27, 83 (1958).