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Stoicheiometry of oxidative phosphorylation with tetramethyl-*p*-phenylenediamine in rat-liver mitochondria

JACOBS[1] introduced the use of tetramethyl-*p*-phenylenediamine (TMPD) as a substrate for the study of oxidative phosphorylation in the cytochrome *c* oxidase region of the respiratory chain. With catalytic amounts of TMPD, kept reduced by ascorbate, P:O ratios of approx. 1.0 could sometimes be obtained with rat-liver mitochondria. P:O ratios equal to or exceeding 1.0 were subsequently reported by other investigators[2–6].

MINNAERT AND VAN KAMMEN-WERTHEIM[7] first pointed out the errors that could be caused by neglecting the contribution of endogenous substrate in estimating the P:O ratio associated with terminal electron transport. HOWLAND[3] considered that endogenous substrate played no role under his experimental conditions in which he obtained P:O ratios with TMPD exceeding 1.0, and suggested that two phosphorylation sites, one of which is antimycin-sensitive, may occur in the span TMPD–oxygen. Other investigators[4–6,8] have contested this and have proposed that the high P:O ratios measured are due to the simultaneous oxidation of endogenous substrate, either directly *via* all the components of the respiratory chain[5,6,8], or, in the presence of antimycin, *via* a TMPD shunt by-passing the antimycin block[4,5].

However, no detailed study of the P:O ratio associated with the oxidation of TMPD, in the absence of oxidation of endogenous substrate, has been reported. For this reason, we have carried out measurements in the presence of arsenite or rotenone to minimize any contribution from endogenous substrate. In order to estimate directly any residual contribution, we have measured not only oxygen uptake and phosphate esterification, but also the disappearance of the ascorbate used to keep the TMPD in the reduced state (Table I).

In each experiment, parallel incubations were carried out with heart-muscle preparation. Since heart-muscle preparation contains no endogenous substrate, a $\Delta O:\Delta$ascorbate ratio of 1.00 would be expected. The mean value of 1.04 $\pm$ 0.02 (S.E.) obtained is a measure of the intrinsic analytical errors*. Since with rat-liver mitochondria a mean $\Delta O:\Delta$ascorbate ratio of 1.05 $\pm$ 0.02 was obtained, it may be concluded that under our experimental conditions there is no contribution of endogenous substrate to the oxygen uptake in the presence of TMPD–ascorbate. The mean P:O ratio of 0.94 $\pm$ 0.02 (or P:Δascorbate of 0.99), therefore, must represent the phosphorylation coupled with the oxidation of TMPD. Several investigators[3,5,9,14,15] have indicated that TMPD reacts with the respiratory chain at the level of cytochrome *c*. On the other hand, it is believed that only one phosphorylation site is involved in the cytochrome *c* oxidase region of the respiratory chain[16]. The value of 0.94 for the P:O ratio obtained in our experiments, although less than those (1.10–1.18) reported by HOWLAND[3], is therefore unexpectedly high in view of the relatively poor respiratory control measured with this substrate. Our results suggest that although there is only one phosphorylation site in the terminal region of the respiratory chain,

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

* In the reaction mixture used, no oxygen uptake or ascorbate disappearance was observed in the absence of mitochondria or in heart-muscle preparation.

TABLE I

STOICHEIOMETRY OF OXYGEN UPTAKE, ASCORBATE DISAPPEARANCE AND PHOSPHATE ESTERIFICATION IN THE PRESENCE OF TMPD IN RAT-LIVER MITOCHONDRIA

The reaction mixture (final volume, 1 ml; final pH, 7.5) contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl, 20 mM P_i, 0.5 mM ADP, 20 mM glucose, 25 mM sucrose, 5 I.U. hexokinase, rat-liver mitochondria[9] (2–4.6 mg protein) and either 1 mM arsenite or 1 μg rotenone. The concentration of TMPD was 240 μM in Expt. 3 and 60 μM in all other experiments. The concentration of ascorbate was 5–10 mM. The reaction was carried out for 16–20 min in Warburg flasks at 25°. The reaction was stopped with 5% trichloroacetic acid. Phosphate esterification was measured as described in ref. 10. Ascorbate was measured by titration with 2,6-dichlorophenolindophenol[11], the latter being standardized with standard $Na_2S_2O_3$ (see ref. 12). In each experiment parallel incubations with heart-muscle preparation[13] (0.02–0.14 mg protein/vessel) were carried out. The reaction mixture contained the same components as with rat-liver mitochondria, except that hexokinase was absent and 13 μM cytochrome *c* was present. The ascorbate and TMPD solutions were prepared immediately before use in 2 mM EDTA and neutralized. Each value is the mean of two incubations.

Expt. No.	*Heart-muscle preparation*			*Rat-liver mitochondria*				
	ΔO (μatoms)	ΔA ascorbate (μmoles)	$\Delta O:\Delta A$ ascorbate	ΔO (μatoms)	ΔA ascorbate (μmoles)	$\Delta O:\Delta A$ ascorbate	Δ Esterified P (μmoles)	P:O
	Arsenite present							
1	3.36	3.19	1.05	3.81	3.56	1.08	3.46	0.91
2	3.23	3.26	0.99	3.69	3.46	1.07	3.76	1.02
3	3.56	3.27	1.09	5.29	5.38	0.98	4.92	0.94
			Mean 1.05			Mean 1.04		Mean 0.95
	Rotenone present							
4	4.42	4.35	1.02	4.17	4.22	0.99	3.91	0.94
5	1.89	1.90	1.00	5.04	5.16	0.98	4.36	0.87
6	6.43	5.91	1.09	5.60	4.89	1.15	5.21	0.94
7	6.56	6.79	0.97	6.43	6.33	1.02	5.85	0.91
8	4.99	4.62	1.08	5.28	4.74	1.11	5.00	0.95
			Mean 1.03			Mean 1.05		Mean 0.92
Mean of all experiments			1.04 ± 0.02*			1.05 ± 0.02*		0.94 ± 0.02*

* Standard error of mean.

another reaction leading to the synthesis of ATP occurs under our experimental conditions, as originally proposed by HOWLAND[3].

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Laboratory of Biochemistry,*
B.C.P. Jansen Institute,
University of Amsterdam,
Amsterdam (The Netherlands)

R. A. F. M. CHAMALAUN
J. M. TAGER

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* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.