Measurement of the P:O ratio associated with the oxidation of ascorbate by rat-liver mitochondria

It is the purpose of the present report to draw attention to a source of error which can arise under certain circumstances in the measurement of the P:O ratio associated with the oxidation of ascorbate by rat-liver mitochondria. This is caused by the presence of appreciable amounts of endogenous substrate in these mitochondrial preparations[1].

The experiments described were carried out with rat-liver mitochondria isolated by the method of HOGEBOOM[2], exactly as described by MYERS AND SLATER[3]. The suspension in 0.25 *M* sucrose was diluted 3.3-fold with ice-cold distilled water, to give a suspension medium of 0.075 *M* sucrose (see ref. 4) and allowed to stand 10 min at 0° before adding to the reaction medium. The final composition of the reaction medium (volume 1 ml) was 25 m*M* sucrose, 10 m*M* potassium phosphate, 30 m*M* glucose, 0.5 m*M* ADP, 5 m*M* $MgCl_2$, 2 m*M* EDTA, 20–50 m*M* ascorbate (freshly prepared), 100–150 units[5] crystalline yeast hexokinase[6], and varying amounts of cytochrome *c*. The final pH was 6.5. When ascorbate was omitted, 40 m*M* sucrose was added to maintain the tonicity of the solution. When antimycin was added, the reaction mixture contained 0.5 % ethanol derived from the antimycin solution. The cytochrome *c*, prepared by the method of MARGOLIASH[7], had $A_{550m\mu}$ (reduced)/$A_{280m\mu}$ (oxidized) of 1.22 or higher. Oxidative phosphorylation was measured by the method of SLATER[8]. The O_2 uptake was measured with differential manometers. The constants, calculated according to VAN DORP AND SLATER[9], were between 0.25 and 0.70 μl O_2/mm manometer fluid. The O_2 uptake was corrected for that found in a control experiment, in which mitochondria were omitted.

The hypotonic pretreatment of the mitochondria caused some development of latent ATPase, but in the presence of Mg^{++} dinitrophenol still stimulated the ATPase 12-fold when tested as described by GREENGARD *et al.*[10]. The rate of oxidation of ascorbate was increased 2–3 times by the addition of phosphate or of dinitrophenol. (In the presence of dinitrophenol, phosphate caused no further increase of the rate of oxidation–see BORST AND SLATER[11]). The P:O ratio was not affected by variation of the ADP concentration between 0.24 and 3.2 m*M*.

Table I shows that variation of the cytochrome *c* concentration between 10 and 250 μM had little effect on the P:O ratio. Thus, high concentrations of cytochrome *c* did not appreciably uncouple oxidative phosphorylation under our conditions (see ref. 4, 12). This conclusion was supported by the finding that 100 μM cytochrome *c*, either in the presence or absence of 10^{-4} *M* dinitrophenol, had no effect on the ATPase activity of rat-liver mitochondria (see ref. 3).

In Fig. 1A, the results are given of an experiment, in which the amount of esterified P was followed as a function of time, both in the absence (curves 1 and 3) and presence (curves 2 and 4) of antimycin. Curves 1 and 2 describe the results obtained in the presence of ascorbate and cytochrome *c*, curves 3 and 4 the corresponding results in the absence of substrate. Curve 3 shows that there was considerable esterification of phosphate when the mitochondria were incubated in the reaction medium, in the absence of added substrate, much of the esterification occurring in the first

Abbreviations: ADP, ATP, adenosine diphosphate and triphosphate; EDTA, ethylenediamine tetraacetic acid.

TABLE I

EFFECT OF CYTOCHROME C CONCENTRATION ON P:O RATIO OF RAT-LIVER MITOCHONDRIA

Conditions as described in text; 0.45–0.65 μg antimycin/mg protein.

Cytochrome c (μM)		*P:O*		
		Expt. 1		*Expt. 2*
	Ascorbate (mM)	*20*	*50*	*20*
9		0.51	0.62	0.46
12		0.54	0.59	0.45
22			0.64	0.46
43		0.54	0.65	0.45
62		0.45	0.59	0.46
124		0.46	0.55	
248		0.45	0.54	

5 min. It was abolished by addition of 0.45 μg antimycin/mg protein (curve 4), an amount of antimycin which was sufficient to inhibit the oxidation of succinate by 98 %. This esterification of phosphate in the absence of substrate is very likely due to endogenous substrates present in the mitochondria[1].

Since oxidative phosphorylation associated with the oxidation of endogenous substrates has a higher P:O ratio than that associated with the oxidation of ascorbate[13], the latter P:O will be over-estimated unless interference due to oxidation of endogenous substrate is eliminated or allowed for. With the method used in this study to measure the O_2 uptake, the error is even greater than it appears, because most of the extra O_2 uptake occurs in the first 5 min, and is not detected or allowed for in our procedure in which the uptake in the first 5 min is calculated by extrapolation, assuming a constant oxidation rate. The rate of phosphorylation in the first 2 min is about double that between 5 and 25 min. This could give the impression that there is a very labile phosphorylation step associated with the oxidation of ascorbate

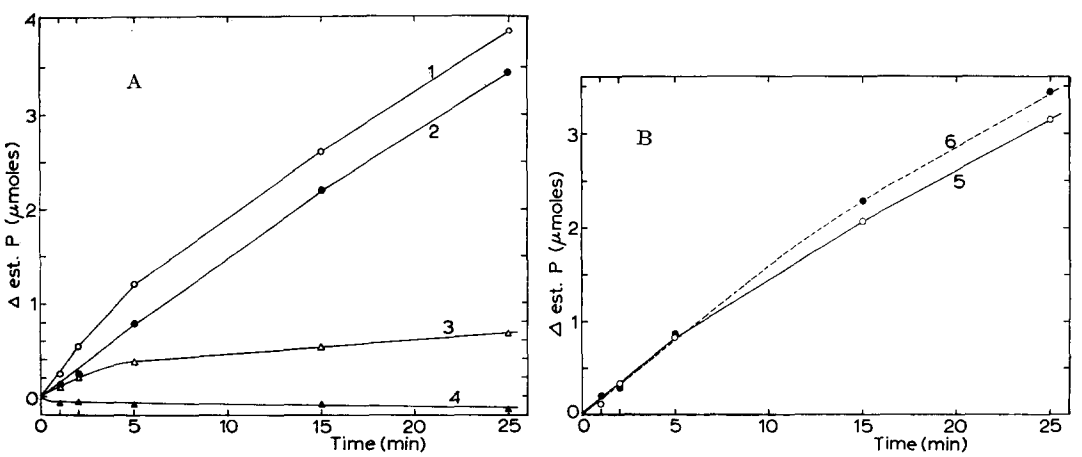

Fig. 1. Phosphorylation coupled to oxidation of endogenous substrate and to oxidation of added ascorbate. Protein, 2.20 mg/ml. A. Curve 1, ascorbate, 0.02 *M*; cytochrome *c*, $2.2 \cdot 10^{-5}$ *M*; curve 2, same as curve 1 with addition of antimycin (0.45 μg/mg protein); curve 3, no cytochrome *c* or ascorbate; curve 4, same as 3 with addition of antimycin (0.45 μg/mg protein). B. Curve 5, curve 1 of A *minus* curve 3; curve 6, curve 2 of A *minus* curve 4.

and, moreover, that this labile step is sensitive to antimycin (*cf.* curves 1 and 2, Fig. 1A).

When, however, the amount of esterified P found in the absence of ascorbate and cytochrome *c* is subtracted from that found in the presence of substrate, practically identical values were obtained in the presence and absence of antimycin (Fig. 1B), and there was little fall in oxidative phosphorylation during the course of the experiment.

Unless oxidation of endogenous substrate is inhibited by the addition of antimycin, or a suitable correction is made, the measured P:O ratio will be increased whenever conditions are altered in such a way as to increase the proportion of the total O_2 uptake which is concerned with the oxidation of endogenous substrate, *e.g.* by running the experiment for a short time, or by choosing conditions unfavourable to the oxidation of ascorbate, such as the use of mitochondria which have not been subjected to hypotonic pretreatment. The finding of increased P:O ratios under these conditions will lead to the conclusion, which might be incorrect, that these conditions favour the phosphorylating step in the cytochrome oxidase region of the respiratory chain.

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Endogenous respiration of rat-liver mitochondria*

The presence of appreciable amounts of endogenous substrate in rat-liver mitochondria was first reported by CHANCE AND WILLIAMS[3]. During the course of measurements of the phosphorylation coupled to oxidation of ascorbate in the presence of cytochrome *c* and rat-liver mitochondria, we found that the simultaneous oxidation of

Abbreviations: ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid.

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