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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[1] K. MINNAERT, *Biochim. Biophys. Acta*, 44 (1960) 595.
[2] G. H. HOGEBOOM, *Methods in Enzymology*, Vol. I, Academic Press, New York 1955, p. 16.
[3] D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558, 572.
[4] A. L. LEHNINGER, M. HASSAN AND H. C. SUDDUTH, *J. Biol. Chem.*, 210 (1954) 911.
[5] L. BERGER, M. W. SLEIN, S. P. COLOWICK AND C. F. CORI, *J. Gen. Physiol.*, 29 (1946) 379.
[6] R. A. DARROW AND S. P. COLOWICK, personal communication.
[7] E. MARGOLIASH, *Biochem. J.*, 56 (1954) 529, 535.
[8] E. C. SLATER, *Biochem. J.*, 53 (1953) 157. 521.
[9] A. VAN DORP AND E. C. SLATER, *Biochim. Biophys. Acta*, 33 (1959) 559.
[10] P. GREENGARD, K. MINNAERT, E. C. SLATER AND I. BETEL, *Biochem. J.*, 73 (1959) 637.
[11] P. BORST AND E. C. SLATER, *Nature*, 184 (1959) 1396.
[12] C. COOPER AND A. L. LEHNINGER, *J. Biol. Chem.*, 219 (1956) 519.
[13] B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 383.

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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.

* This paper and the previous[2] is part of the Ph.D. thesis of the author, published in Dutch[1]. It was defended in public on June 22nd 1960, before the Senate of the University of Amsterdam.

endogenous substrate was a disturbing factor[1,2]. This paper reports a further study of the rate of oxidation of endogenous substrate and of the accompanying phosphorylation.

The oxidation of endogenous substrate was followed by measuring either the oxygen uptake or the esterification of inorganic phosphate. The experimental methods were the same as those described in the accompanying paper[2]. The "standard reaction mixture" consisted of phosphate, 10 mM; glucose, 30 mM; ADP, 0.6 mM; $MgCl_2$, 5 mM; EDTA, 2 mM; sucrose, 65 mM; and hexokinase, 100–150 units. The reaction mixture was brought to pH 6.5. The mitochondria were subjected to a hypotonic treatment (7.5 mM sucrose) for 10 min at 0°.

The esterification of inorganic phosphate during aerobic incubation of the mitochondria in the reaction mixture is a measure of the oxidation of substrates inside the mitochondria or adsorbed on their surface. The hypotonic pretreatment of the mitochondria or an extra washing with 0.25 M sucrose, had little effect on the amount of endogenous substrate measured in this way (see Fig. 1).

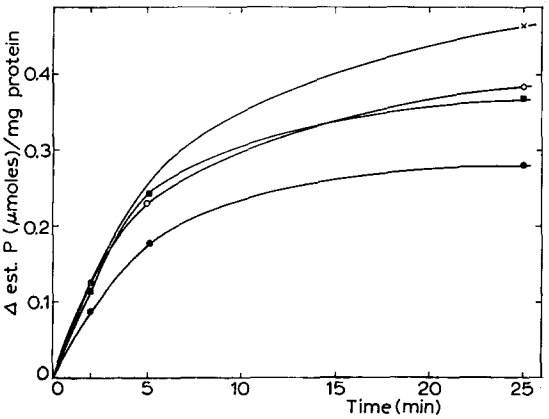

Fig. 1. Effect of hypotonic pretreatment and of washing on amount of endogenous substrate in rat-liver mitochondria. ×, normal mitochondria; ○, normal mitochondria given extra wash; ■, hypotonically pretreated mitochondria (normal preparation used in this study); ●, mitochondria given extra wash and then hypotonically pretreated.

The amount of phosphate esterified in the first 5 min was 0.20 μmole/mg protein (average of 10 experiments; range, 0.14–0.25). After 5 min the rate falls off; over the first 25 min, 0.36 μmole P/mg protein were esterified (average of 7 experiments; range, 0.28–0.46). If we assume a P:O ratio of 2, this corresponds to a Qo_2 of 13.5 during the first 5 min and of 2.6 during the following 20 min. It is not possible to indicate the total amount of endogenous substrate since the oxygen uptake does not come to an end even after several hours.

The maximal initial rate of phosphorylation is obtained with the standard reaction mixture. When ADP was omitted, the initial rate was much slower and a burst of activity equal to the initial rate was found when ADP was added after 5.5 min (Fig. 2). This shows that the concentration of ADP limits the rate of phosphorylation in the presence of phosphate, hexokinase and glucose.

The oxygen uptake by endogenous substrates is inhibited by malonate (see ref. 3, 4) and arsenite. Monoiodoacetate is also inhibitory. The degree of inhibition

by this compound (25 % at 0.2 m*M* and 70 % at 1 m*M*) points to an inhibition in the Krebs cycle, rather than in the glycolytic reactions[5]. The preparation of serum albumin used (Armour Laboratories, fraction V) increased the oxygen consumption after 5 or 25 min by a factor of 4, when 10 mg/ml were added to the reaction mixture. However, the supernatant of boiled serum albumin had the same effect while dialysed serum albumin was inactive.

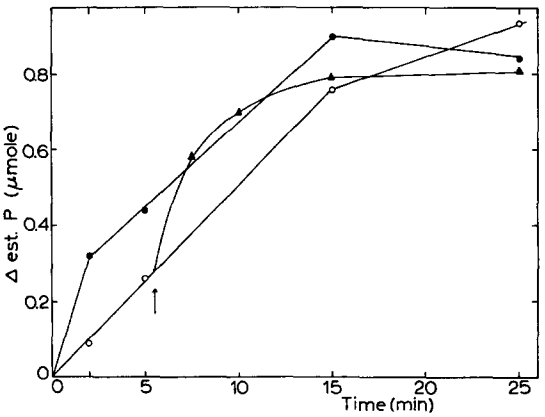

Fig. 2. Effect of ADP on phosphorylation coupled to oxidation of endogenous substrate. ●, standard reaction mixture; ○, ADP omitted; ▲, ADP added after 5.5 min.

The amount of oxygen taken up in 4 h was about 0.75 μatom/mg protein or 75 μatoms/ml wet wt. mitochondrial pellet. If we assume that the endogenous substrate is fatty acid[4] which is oxidized to CO_2 and H_2O, this amount of oxygen is equivalent to a concentration of 1.5 m*M* stearic acid in the pellet. Since it is unlikely that such high concentrations of a substrate are present in mitochondria, it seems probable that the endogenous substrate is continuously formed, *e.g.* by hydrolysis of lipid.

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[1] K. MINNAERT, *Over cytochroom* c *oxydase*, Academic thesis, Amsterdam, 1960. V.R.B. Kleine der A 3–4, Groningen, 1960.
[2] K. MINNAERT AND A. R. VAN KAMMEN-WERTHEIM, *Biochim. Biophys. Acta*, 44 (1960) 593.
[3] B. CHANCE AND G. R. WILLIAMS, *J. Biol. Chem.*, 217 (1955) 383.
[4] M. KLINGENBERG, W. SLENCZKA AND E. RITT, *Biochem. Z.*, 332 (1959) 47.
[5] W. C. YANG, *Science*, 125 (1957) 1087.

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