RESPIRATORY CHAIN LINKED H_2O_2 PRODUCTION IN PIGEON HEART MITOCHONDRIA

G. LOSCHEN and L. FLOHÉ
Physiologisch-Chemisches Institut der Universität Tübingen, 7400 Tübingen, W. Germany

and

B. CHANCE
Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104, USA

Received 24 August 1971

1. Introduction

It has been known for many years that the succinate dehydrogenase of the intestinal nematode *Ascaris lumbricoides* reacts with molecular oxygen without participation of the cytochrome system under formation of H_2O_2 [1–3]. Besides, P.K. Jensen reported that at least part of the antimycin insensitive respiration of beef heart ETPs might be due to H_2O_2 formation [4]. It was assumed that NADH was the autoxidable component of the respiratory chain responsible for the H_2O_2 generation. P. Hinkle et al. [5] presented evidence for an ATP dependent H_2O_2 formation on the substrate side of cytochrome *b* in submitochondrial particles of beef heart. But all these results require experimental corroboration because no method had been available for determining low concentrations of H_2O_2 in biological material directly. Recently, Chance and Oshino [6] approached this problem in the following way: preparations of rat liver mitochondria were observed with a dual wavelength spectrophotometer. Formation of compound I of peroxisomal catalase present in these preparations was considered indicative of H_2O_2.

In the present study H_2O_2 was determined by the horse radish peroxidase dependent reaction of H_2O_2 with the fluorescent dye scopoletine [7]. A decrease of fluorescence intensity demonstrates directly the rate of formation of H_2O_2 available in the extramitochondrial medium. By application of this method we have found that under aerobic conditions H_2O_2 is generated in pigeon heart mitochondria only with succinate but not with NADH linked substrates. The site of H_2O_2 generation in the respiratory chain could be localized between cytochrome *b* and succinate dehydrogenase. Formation of H_2O_2 apparently depends on the degree of reduction of the respiratory chain, since it only occurs in state 4 or, in uncoupled mitochondria, in the presence of antimycin.

2. Materials and methods

Scopoletine was purchased from Mann Research Company, New York, horse radish peroxidase (HPR), grade II, from Boehringer, Mannheim. All other reagents were products of Sigma Chem. Co.

About 0.2 ml freshly prepared pigeon heart mitochondria [8] (30 mg protein/ml) were added to a quartz cuvette containing 2 ml of oxygen saturated MS-MOPS buffer (0.22 M mannitol/0.75 M sucrose/ 0.0005 M EDTA/0.020 M morpholine propane sulfonate, pH 7.0). All other reagents were added in small portions of concentrated solutions, as indicated in the figures (see below). The fluorescence measurements were carried out in a Hitachi Perkin Elmer Spectro-Fluorometer (excitation 350 nm,

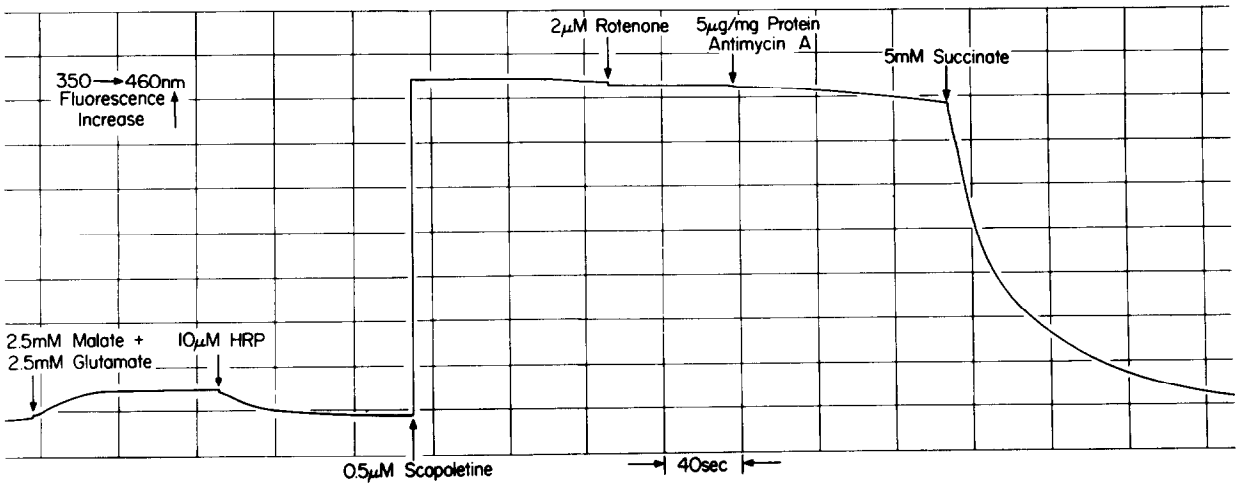

Fig. 1. Formation of H_2O_2 in a suspension of pigeon heart mitochondria. The arrows show addition of reagents. The final concentration of reagents is given in the figure. Decrease of scopoletine fluorescence indicates formation of H_2O_2.

emmision 460 nm). The scopoletine concentration was adjusted in such a way that its fluorescence exceeded the substrate induced fluorescence at least tenfold (see fig. 1). Any marked decrease in fluorescence intensity therefore demonstrates an oxidation of scopoletine by H_2O_2 via HPR.

Protein was determined by the biuret method [9].

3. Results

As shown in fig. 1, addition of malate and glutamate to the suspension of mitochondria causes a slight increase of fluorescence intensity primarily due to reduction of pyridine nucleotides. After addition of HPR the fluorescence decreases to the initial level for unknown reasons. At a concentration of 0.5 μM scopoletine a high fluorescence intensity is observed which remains constant even after addition of rotenone. Since the presence of H_2O_2 would have resulted in a decline of the scopoletine fluorescence, we can conclude that neither in the presence nor in the absence of rotenone any marked net formation of H_2O_2 is induced by NADH linked substrates in intact pigeon heart mitochondria.

The addition of succinate, however, entails a rapid decrease of fluorescence intensity which reflects the rate of H_2O_2 formation. A decrease of scopoletine fluorescence after addition of succinate does also occur in the absence of antimycin (see figs. 2 and 3), although apparently at a lower rate (compare fig. 1 with figs. 2 and 3). The lack of any significant H_2O_2 formation by malate and glutamate even in the presence of antimycin shows that no considerable amount of succinate is built up through the Krebs cycle under our experimental conditions. The fluorescence decrease after addition of succinate in fig. 1 reveals that the detection of H_2O_2 by the scopoletine method is feasable even in the presence of a large excess of reducing equivalents.

Summarising the results of the experiment shown in fig. 1, we can state that generation of H_2O_2 in intact pigeon heart mitochondria is linked to a succinate dependent reaction. The site of H_2O_2 production can tentatively be localized between succinate dehydrogenase and the antimycin block.

The following experiment (fig. 2) concerns the question whether the mitochondrial H_2O_2 production depends on the metabolic state of the organelles. H_2O_2 production is observed in state 4 in the presence of succinate and phosphate. When 0.7 mM ADP is added, H_2O_2 formation immediately stops (state 4 → state 3 transition [10]). After consumption of ADP (state 3 → state 4 transition) H_2O_2 production starts again at about the same rate as before.

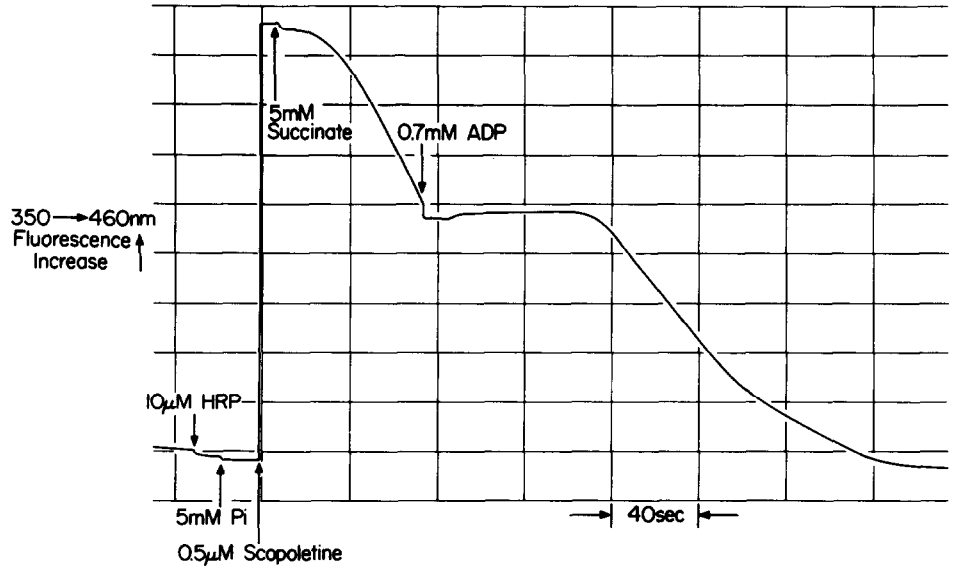

Fig. 2. Influence of the metabolic state of pigeon heart mitochondria on succinate dependent H_2O_2 formation. For further explanations see text.

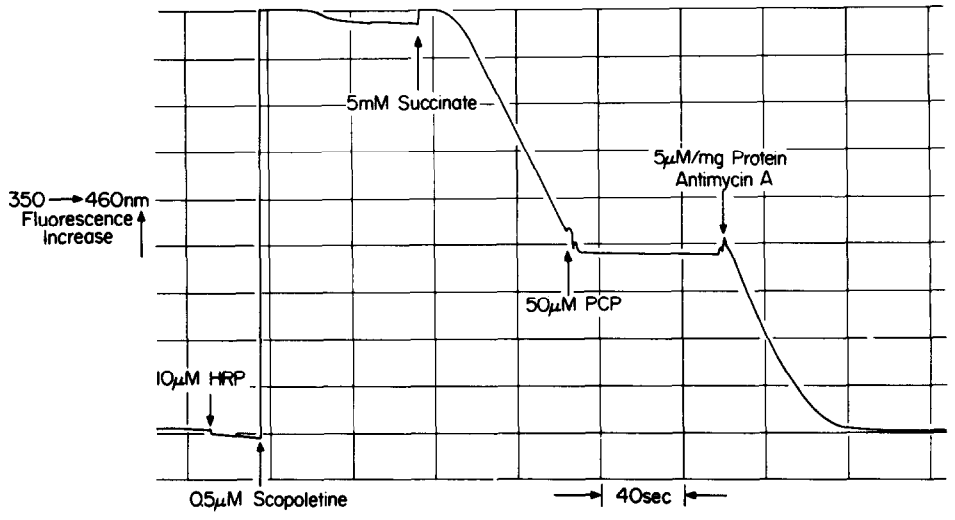

Fig. 3. Influence of uncoupler and inhibitor of the respiratory chain on H_2O_2 formation in pigeon heart mitochondria. For further explanations see text.

A possible explanation for this phenomenon is suggested by the experiment shown in fig. 3: succinate dependent H_2O_2 formation of the respiratory chain is completely prevented by the uncoupling agent pentachlorophenol (PCP). In the uncoupled mitochondria, however, H_2O_2 formation is restored if respiration is inhibited by antimycin A. The common feature of the two metabolic states in which H_2O_2 formation is observed is the high degree of reduction on the substrate side of the antimycin block.

4. Discussion

As pointed out by Perschke and Broda [11], the scopoletine method is a proper tool for detecting traces of H_2O_2 in biological material. Some restrictions, however, have to be considered if this method is employed in a complex system like a suspension of mitochondria: 1) The change of fluorescence intensity conditioned by the reaction of scopoletine with H_2O_2 must largely exceed the variance of fluorescence due to the metabolism of the mitochondria. This demand was adequately satisfied in our experiments. (It should be mentioned that scopoletine at the concentration of 0.5 μM does not exhibit any uncoupling or inhibitory effect on pigeon heart mitochondria.) 2) Efficient donors of HPR should be absent in the reaction medium. Otherwise, the decrease of scopoletine fluorescence would not represent the actual rate of H_2O_2 formation. 3) The determination covers only H_2O_2 present in the extramitochondrial medium. 4) H_2O_2 destruction by alternative pathways must be negligible.

Considering the last three points, the formation of H_2O_2 observed in our experiments can only be regarded as the lowest limit of the actual reaction rate. Besides, it is probably the absence of catalase and GSH-peroxidase which enables a direct determination of H_2O_2 in pigeon heart mitochondria. (No activity of either enzyme could be determined at a protein concentration of 2.2 mg/ml).

As mentioned earlier, the formation of H_2O_2 in pigeon heart mitochondria by succinate presupposes a high degree of reduction of the respiratory chain. Corresponding conclusions were drawn by Chance and Oshino from experiments with rat liver mitochondria [6]. The mechanism of this process needs further investigation. It is not unlikely, however, that under special metabolic conditions the completely reduced flavine component of succinate dehydrogenase is oxidized by molecular oxygen. In agreement with this concept, Bueding and Charms [1] assume that H_2O_2 formation in the abnormal respiratory chain of ascaris results from autoxidation of succinate dehydrogenase. In addition, Thurman [12] recently showed that H_2O_2 is produced by a flavoprotein of the microsomal fraction.

The biological significance of our results represents a serious problem. It was demonstrated that in the perfused rat liver the mitochondria approach state 4 [13]. Therefore, the possibility of a respiratory chain linked H_2O_2 production under physiological conditions must be considered. In rat liver, however, there are two enzymes which destroy H_2O_2 at a sufficient rate: (1) GSH-peroxidase in the matrix space of mitochondria and in the soluble fraction [14, 15], and (2) catalase in the peroxisomes [16]. But no significant activity of either enzyme could be found in pigeon heart mitochondria. There might be a third unknown pathway of H_2O_2 destruction in heart. This assumption, however, is quite unlikely because H_2O_2 definitively accumulated in our experiments when succinate was added prior to HRP. Alternatively, we have to assume that there is no need to eliminate H_2O_2 because state 4 is never reached in the mitochondria of pigeon heart *in vivo*.

Acknowledgements

Many stimulating discussions with Dr. N. Oshino, are gratefully acknowledged. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (Fl 61/2).

References

[1] E. Bueding and B. Charms, J. Biol. Chem. 196 (1952) 615.
[2] E. Kmetec and E. Bueding, J. Biol. Chem. 236 (1961) 584.
[3] K.S. Cheah and B. Chance, Biochim. Biophys. Acta 223 (1970) 55.
[4] P.K. Jensen, Biochim. Biophys. Acta 122 (1966) 157.
[5] P.C. Hinkle, R.A. Butow, E. Racker and B. Chance, J. Biol. Chem. 242 (1967) 5169.
[6] B. Chance and N. Oshino, Biochem. J. 122 (1971) 225.
[7] W.A. Andreae, Nature 175 (1955) 859.
[8] B. Chance and B. Hagihara, in: Proc. 5th Internatl. Congress Biochem. Vol. 5, Moscow, 1963, ed. A.N.M. Sissakian (Pergamon Press, New York, 1963) p. 3.
[9] A.G. Gornall, C.J. Bardawill and M.M. David, J. Biol. Chem. 177 (1949) 751.
[10] B. Chance and G.R. Williams, in: Advances in Enzymology, Vol. 17, ed. F.F. Nord (Interscience Publishers, Inc., New York, 1956) p. 65.
[11] H. Perschke and E. Broda, Nature 190 (1961) 257.
[12] R. Thurman (unpublished results).
[13] R. Scholz, R.G. Thurman, J.R. Williamson, B. Chance and T. Bücher, J. Biol. Chem. 244 (1969) 2317.
[14] L. Flohé and W. Schlegel (in preparation).
[15] L. Flohé, Klin. Wschr. 49 (1971) 669.
[16] F. Leighton, B. Poole, H. Beaufay, P. Baudhuin, J.W. Coffey, S. Fowler and C. De Duve, J. Cell Biol. 37 (1968) 482.