LOSS AND MAINTENANCE OF ENERGY-LINKED FUNCTIONS IN AGED MITOCHONDRIA

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Summary. A number of energy-linked functions were studied in mitochondria aged at 2° for 0-14 days. The stimulation of respiration by ADP, by dinitrophenol (DNP), by Ca^{++}, or by K^+ in presence of valinomycin, the ATP-supported active transport of Ca^{++}, and the succinate-induced reversal of the electron flow, disappeared after 2-4 days. The response of the respiration to ADP was slightly more sensitive to aging than most of the other energy-linked functions. The ATP-induced, phosphatidyl (PI) -dependent contraction of mitochondria after large amplitude swelling was on the other hand still demonstrable after 10-14 days of aging.

In addition to the synthesis of ATP, isolated mitochondria carry out a number of energy-linked reactions. Among them, the reversal of the respiratory chain (Chance and Hollunger, 1957; Klingenberg and Von Hafen, 1963; Low and Vallin, 1963), the reduction of NADP by NADH (Danielson and Ernster, 1963; Ernster and Lee, 1964), the active uptake of various ions (Lehninger et al., 1967), and the ATP-induced contraction following large amplitude swelling (Lehninger, 1962). Energy rich compounds (or states) generated by either the coupled operations of the respiratory chain or by the hydrolysis of ATP have been suggested to energize these reactions. The present study describes experiments on the loss of energy-linked functions during aging of mitochondria. It has been found that most of the energy-linked functions disappear after 2-4 days. However, the ATP-linked contraction after large amplitude swelling can still be demonstrated after 10-14 days. The results suggest that aging inactivates a step in the energy-coupling sequence located between the oligomycin-sensitive site and the respiratory chain.

METHODS

Mitochondria were prepared from the livers of Wistar strain rats by the procedure of Scheider (1957). Suspensions containing 50 mg of protein per ml were aged at 2°. The stimulation of respiration by ADP, Ca^{++}, DNP, was studied in a medium containing 225 mM mannitol, 60 mM sucrose, 10 mM Tris-Cl, pH 7.2, 10 mM inorganic phosphate (MSTP), 20 mM Na-succinate, and 5 mg of mitochondrial protein in a volume of 2 ml, at 25°. For the valinomycin-induced stimulation of respiration, 40 mM KCl was included in the medium. ATP-supported $^{45}Ca^{++}$ uptake was measured by Millipore filtration, 1 min. after the addition of 400 µM $^{45}Ca^{++}$ to the MSTP medium, containing 5mM ATP, 40 µg Antimycin A, and 12.5 mg of mitochondrial protein; the volume was 5 ml, and the temperature 25°. Succinate-induced reversal of the respiratory chain was measured as described by Ernster and Lee (1967), and large amplitude swelling and ATP-driven contraction according to Lehninger (1962).

RESULTS AND DISCUSSION

Under the conditions used in the present study, the stimulation of respiration by ADP became less evident as aging progressed , until it disappeared almost completely after about 72 hours (Table 1). However, in the presence of bovine serum albumin (BSA) and EDTA it could be demonstrated for longer times, up to a maximum of about 80-96 hours. The stimulation of respiration by DNP or by Ca^{++}, or by K^+ in presence of valinomycin, was on the other hand still routinely demonstrable after 72 hours of aging (Table 1), and in the presence of BSA (Ca^{++}), or of BSA and EDTA (DNP), after 96-120 hours. Some variability was experienced in the time of disappearance of the various energy-linked reactions. Indeed, rather

TABLE 1

Hours of aging	Stimulation of respiration* by				ATP-driven Ca^{++} upt.**	Reversal***
	ADP	DNP	Ca^{++}	Valino.		
1	5.0	7.5	5.8	4.3		
24	2.1	4.6	4.5	3.5		
48	1.6	3.6	3.2	2.1		
72	1.0	2.8	2.6	1.8		
96	1.0	1.0	1.0	1.0		
1	3.6				42.5	32.1
24	2.2				18.3	14.0
48	1.0				0	0

*State 3/State 4; **n Moles Ca^{++}/mg prot./min.; ***n Moles NADH formed/mg prot/min.

often the response to ADP had disappeared already after 48 hours, and that to DNP and to Ca^{++} after 72 hours. The second experiment of Table 1 was carried out on one of these preparations : the reversal of the respiratory chain, the ATP-driven uptake of Ca^{++}, and the response to ADP had already disappeared after 48 hours.

The response to ADP therefore seems slightly more sensitive to aging than some of the other energy-linked reactions. Its delayed disappearance in the presence of BSA suggests the involvement of fatty acids in the early loss of the response. Possibly, fatty acids inhibit the ATP/ADP permease, as has been indicated by Wojtczak and Zaluska (1967).

The ATP-driven contraction of mitochondria behaved differently from the other energy-linked reactions. As shown already by Vignais *et al.* (1964), it disappeared after 48-72 hours of aging, and it was restored specifically by the addition of PI. We have extended these observations, and found that the PI-dependent, ATP-driven contraction was still routinely demonstrable after 10-14 days of aging; i. e., long after the loss of all the other energy-linked reactions (Figure 1). Attempts to restore the other energy-linked reactions in aged mitochondria with PI were on the other hand completely unsuccessful. The unexpected observation was also made that

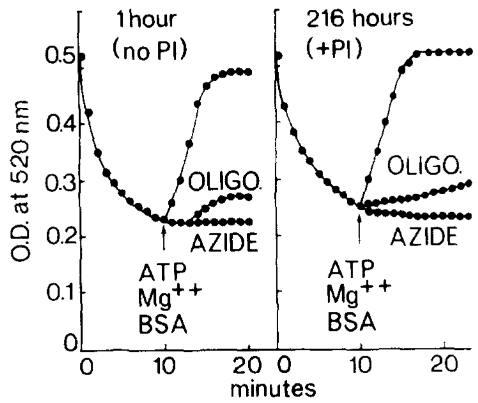

Figure 1. ATP-supported contraction of aged mitochondria. Experimental details are described in the Methods. Final volume, 5 ml, temperature, 21°. Swelling was accelerated with oleate. Concentrations: protein, about 1 mg, PI, about 0.1 mM, Mg Cl_2, 3 mM, ATP, 3 mM, BSA, 10 mg, oleate 0.01 mM, oligomycin, 2 µg, azide, 2 mM.

the PI-independent contraction reappeared after 4-5 days of aging, became progressively more evident, and disappeared completely after 10-14 days. This puzzling phenomenon is currently under investigation.

As in fresh mitochondria, oligomycin and azide completely abolished the PI-dependent contraction. The sensitivity of the contraction to oligomycin suggests the involvement of phosphorylated intermediates, which can thus apparently be formed also in extensively aged mitochondria; this conclusion is supported by the observation that mitochondria retain oligomycin sensitive ATPase after prolonged aging (Carafoli and Gazzotti, in preparation). On the other hand, the ATP-supported Ca^{++} uptake, the response of the respiration to ADP, and the other energy-linked functions disappeared much earlier than the contraction. It is suggested that aging inactivates a step in the energy-coupling sequence located between the oligomycin sensitive site and the respiratory chain. This step is not involved in the ATP-driven contraction, but is involved in all the other energy-linked reactions. The results also imply that the adenine nucleotide permease is either not involved in the ATP-driven contraction of aged mitochondria, or is still functioning after extensive aging. The effects of atractyloside on the contraction of aged mitochondria, and of PI on the ATP/ADP permease are currently under investigation.

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REFERENCES

1. Chance, B., and Hollunger, G., Federation Proc., 16, 163 (1957).
2. Klingenberg, M., and Von Hafen, H., Biochem. Z., 337, 120 (1963).
3. Low, H., and Vallin, I., Biochim. Biophys. Acta, 69, 361 (1963).
4. Danielson, L., and Ernster, L., Biochem. Biophys. Res. Commun., 10, 91 (1963).
5. Ernster, L., and Lee, C.P., Methods in Enzymol., (Edited by R.W. Estabrook and M. Pullman), vol. 10, Acad. Press, p. 738 (1967).
6. Lehninger, A.L., Carafoli, E., and Rossi, C.S., Advances in Enzymol., 29, 259 (1967).
7. Lehninger, A.L., Physiol. Rev., 42, 467 (1962).
8. Schneider, W.C., Manometric Techniques (Edited by W.W. Umbreit, R. Burris and J.F. Stauffer), Burgess, 188 (1957).
9. Wojtczak, L., and Zaluska, H., Biochem. Biophys. Res. Commun., 28, 76 (1967).
10. Vignais, P.V., Vignais, P.M., and Lehninger, A.L., J.Biol. Chem., 239, 2011 (1964).