Table 3. Restoration of aging effect. Time of aging: 60 minutes. Additions: AMP, ADP and ATP, 2.4 mM; Mn++, 0.5 mM. Values referred to mitochondria derived from 100 mg liver.

System	Additions	μmoles DPNH/5min
intact	—	0.62
aged	—	2.31
	AMP	2.24
	ADP	1.43
	ATP	0.82
	Mn++	2.35
	ATP+Mn++	0.65

and Mn++ (Table 3). In the latter case, Mn++ alone is without effect, while ATP alone or, to a lesser extent, ADP, but not AMP, may exhibit a certain action.

In the experiment presented in Table 4, it is shown that the effect of ATP and Mn++ is not due to an inhibition of the oxidation of glutamate, but rather to a structural reconstruction of the mitochondrial system, also involving a restoration of the phosphorylative ability.

The results support the previous postulate [1] that the availability of cytoplasmic DPNH is controlled by the structural state of the mitochondria.

1. Ernster, L. *Exptl. Cell Research* **10** (1956) 704, 721.
2. Ernster, L. and Löw, H. *Exptl. Cell Research, Suppl.* **3** (1955) 133.

The Effect of Thyroxine on Mitochondrial Stability

Robert E. Beyer*, Hans Löw and Lars Ernster

Wenner-Gren's Institute, Stockholm, Sweden

It has been demonstrated repeatedly that thyroxine, either administered *in vivo* [1-3] or applied *in vitro* [1,2,4-8], may lower the P/O ratio of isolated rat liver mitochondria. The action of thyroxine applied *in vitro* can be obliterated by Mg++, and more efficiently [7] by Mn++. These ions had been shown previously to protect mitochondria against the action of calcium [9,10]. Using phosphorylative ability [10] and optical density [11] as criteria, it has been shown that under suitable conditions the stability of the enzymic and morphological integrity of isolated mitochondria is an inverse function of the concentration ratio of Ca++/Mg++ and/or Ca++/Mn++ in the system. It was therefore visualized that the action of thyroxine may be reflected primarily in a labilization of mitochondria rather than in a direct action on oxidative phosphorylation. Optical density studies of liver mitochondrial preparations derived from normal and thyroxine treated rats have lent support to this concept [8]. A labilization of the system has been demonstrated to occur even at doses of thyroxine where a direct effect on oxidative phosphorylation could not be ascertained.

All rats used were of the Wistar strain weighing between 125 and 150 g. Hyperthyroidism

* United States Public Health Service Postdoctoral Research Fellow of The National Cancer Institute. Present address: Physiology Dept., Tufts University Medical School, Boston, Massachusetts. U.S.A.

Table 4. DPNH formation and oxidative phosphorylation in aged and restored systems. Additions: DPN, 1.8 mM; ATP, 2.4 mM; Mn++, 0.5 mM. Values referred to mitochondria derived from 100 mg liver.

Aging time minutes	Additions	μmoles DPNH/5 min. (in presence of KCN)	μat. O_2/5 min. (in absence of KCN)	P/O
0	—		1.57	2.84
	DPN	0.88		
30	—		0.45	
	DPN	1.94	1.81	0.33
	DPN, ATP, Mn++	0.69	1.35	1.65
60	—		0.12	
	DPN	2.81	1.09	0.25
	DPN, ATP, Mn++	0.92	1.09	1.86

was induced by intraperitoneal injections of 0.1 mg DL-thyroxine (F. Hoffmann-La Roche and Co. A. G., Basel) for five days, thyrotoxicosis by intraperitoneal injections of 2 mg of DL-thyroxine for various periods. Control animals were injected with suitable sodium chloride solutions. Mitochondria were prepared essentially after Schneider and Hogeboom [12] and suspended in the Mg^{++} free aging medium of Ernster and Löw [10]. Optical density measurements were performed as previously described [11] at room temperature. Phosphorylation was determined according to a modification [13,14] of the Martin and Doty [15] method. Succinate oxidation was measured by the conventional Warburg technique.

Since the *potential* phosphorylative ability of a mitochondrial system may be studied by following the optical density loss during aging in a Mg^{++} free, substrate free, incubation medium supplemented with hexokinase [11], such experiments were performed on liver mitochondria derived from thyrotoxic and control rats. Mitochondria from a thyrotoxic rat liver swell more rapidly than control mitochondria, *i. e.* the former mitochondria become swollen at an earlier stage of the aging process. When the "aging index" is expressed as the time required for 1/2 minimum optical density to be attained, the mean value of 12 experiments for the thyrotoxic mitochondria is 6.5 minutes, for the control, 17.8. The probability that this difference will occur through chance alone is 14 in 10 000. Preliminary data indicate that liver mitochondria from hyperthyroid rats have an aging index intermediate between the control and the thyrotoxic. Further preliminary experiments indicate that the phosphorylation coupled to the oxidation of succinate in mitochondria from thyrotoxic rats possesses a greater sensitivity to calcium ions than does the normal preparation. Table 1 presents an experiment illustrating this point. Phosphorylation became uncoupled from oxidation at the 0.5 mM level in the thyrotoxic series while the P/O ratio remained above 1 at considerably higher levels of calcium in the control flasks. It may also be seen that no uncoupling of oxidative phosphorylation occurred in the thyrotoxic flask when calcium was not added. Unpublished experiments indicate that a preincubation, or equilibration period, may cause a preferential uncoupling of oxidative phosphorylation in the thyrotoxic system, and that this effect may be restored by the addition of ATP and Mn^{++}.

These data are interpreted as follows: Thyroxine, or its active principle, renders the mitochondria, *in vivo*, more susceptible to factors or mechanisms which may lower their phosphorylative efficiency. Upon isolation of the particles and incubation in routinely employed medium this increased susceptibility may elude detection since the incubation media used in studies of oxidative phosphorylation have been systematically developed with the highest possible phosphorylative efficiency as a goal. The lability of the mitochondria, or the potential aging capacity, remains, however. Thus, aging under controlled conditions [10,11], *i. e.* supplying artificial regulatory mechanisms, reveals that the difference between the thyroxine treated and normal animals is the *degree of lability* of the oxidation-phosphorylation systems.

Table 1. Calcium sensitivity of thyrotoxic and control mitochondria.

mM Ca^{++} added	P/O ratios	
	Control	Thyrotoxic
0	1.69	1.67
0.4	1.85	1.24
0.5	2.23	0.16
0.6	1.72	0.10
0.7	1.53	0.06
0.8	1.05	0.03

Each Warburg vessel contained: Mitochondria, 1/20 rat liver; KCl, 150 μmoles; orthophosphate, 50 μmoles; adenylic acid, 4.3 μmoles; glucose, 60 μmoles; sucrose, 125 μmoles; succinate, 30 μmoles; Mg^{++}, 7.5 μmoles; hexokinase, in excess. Final volume, 2.0 ml. Gas phase, air. Temperature, 30° C. Time of incubation, 20 minutes.

1. Martius, C. and Hess, B. *Arch. Biochem.* **33** (1951) 486.
2. Martius, C. in *Hormone und ihre Wirkungsweise*, Springer-Verlag, Berlin, 1955, p. 143.
3. Maley, G. F. and Lardy, H. A. *J. Biol. Chem.* **215** (1955) 377.
4. Hoch, F. L. and Lipmann, F. *Proc. Natl. Acad. Sci.* **40** (1954) 909.
5. Klemperer, H. G. *Biochem. J. London* **60** (1955) 122.
6. Maley, G. F. and Lardy, H. A. *J. Biol. Chem.* **204** (1953) 435.
7. Mudd, S. M., Park, J. H. and Lipmann, F. *Proc. Natl. Acad. Sci.* **41** (1955) 571.
8. Tapley, D. F., Cooper, C. and Lehninger, A. L. *Biochim. et Biophys. Acta* **18** (1955) 597.

9. Lindberg, O. and Ernster, L. *Nature* **173** (1954) 1038.
10. Ernster, L. and Löw, H. *Exptl. Cell Research, Suppl.* **3** (1955) 133.
11. Beyer, R. E., Ernster, L., Löw, H. and Beyer, T. *Exptl. Cell Research* **8** (1955) 586.
12. Schneider, W. C. and Hogeboom, G. H. *J. Biol. Chem.* **183** (1950) 123.
13. Ernster, L., Zetterström, R. and Lindberg, O. *Acta Chem. Scand.* **4** (1950) 942.
14. Lindberg, O., Ljunggren, M., Ernster, L. and Révész, L. *Exptl. Cell Research* **4** (1953) 243.
15. Martin, J. M. and Doty, D. M. *Anal. Chem.* **21** (1949) 965.

The Action of a Mitochondrial Protein Fraction on Mitochondrial ATP-ase and Oxidative Phosphorylation

Robert E. Beyer* and John Glomset

Wenner-Gren's Institute, University of Stockholm, Stockholm, and Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

A protein fraction has been extracted from rat liver mitochondria which stimulates "latent" mitochondrial ATP-ase activity without exhibiting ATP-ase itself and which stimulates the oxidation of succinate in a mitochondrial system containing AMP as the terminal phosphate acceptor. The fraction, in 0.1 M Tris (tris(hydroxymethyl)aminomethane), pH 7.4, has its spectroscopic peak in the Soret region at 414 mμ; in the visible region at 548 mμ and 576 mμ. The mitochondrial ATP-ase stimulating activity is proportional to the concentration of the fraction added.

The mitochondrial fraction (fraction 60) is prepared as follows. Rat liver mitochondria are prepared essentially according to Schneider and Hogeboom [1] using 0.25 M sucrose-0.001 M versene, pH 7.4, as the homogenizing medium. The mitochondrial pellet is washed twice in 0.25 M sucrose and then suspended in Na_2CO_3 which has been adjusted to pH 8.4 with HCl and diluted to 0.1 M. The suspension is then shaken for 12—18 hours at 0—4° C, followed by centrifugation in a Spinco Model L Centrifuge for 7 979 400 *g*-minutes (3 hours, 21 000 RPM, head $\neq$21). The resulting pinkish-yellow supernate is then adjusted to pH 6.5 with acetic acid. The slight cloudy precipitate which forms is centrifuged down. Chilled acetone is added to the supernate in a dropwise fashion with constant stirring to 10 % and maintained in an acetone-dry ice bath made by producing a thick slurry of 9 % acetone and powdered dry ice. The precipitate is spun down in an International Refrigerated Centrifuge at the temperature of the acetone-dry ice bath. Additional fractions are collected at 20, 30, 40 and 60 % acetone, adjusting the acetone-dry ice bath to 19, 29, 39 and 59 % acetone at each subsequent precipitation. The centrifuge is also adjusted accordingly until its lower limit is reached. The pink precipitate resulting from the 40—60 % fraction, called fraction 60, is completely soluble in Tris buffer, pH 7.4, resulting in a cherry red solution. From approximately 100 g of rat liver 10 ml of a solution with an optical density reading at 280 mμ (Beckman DU spectrophotometer) of 1.7 through a 1 cm path length have been obtained. Preliminary experiments indicate that fraction 60 may also be prepared by the above method from "acetone mitochondria" which have been stored *in vacuo* at —15° C for several weeks.

The preparation of a mitochondrial fraction with an action on mitochondrial ATP-ase processes provides a basis for hypotheses which implicate the fraction in oxidative phosphorylation. A protein factor, called mitochrome-I, has been reported briefly [2-4] which appears to have an action similar to fraction 60. Attempts to prepare mitochrome-I, however, have not been successful in our hands. Furthermore, since the only datum of a chemical nature reported for mitochrome-I is its approximate molecular weight, a direct comparison of the two factors has been impossible.

It must be added that fraction 60 is probably not homogeneous. Attempts are in progress to further purify the active principle of fraction 60 by zone electrophoresis and chromatography on cellulose ion exchange columns, as well as to gain some insight into its role in the transfer of high energy phosphate.

* United States Public Health Service Postdoctoral Research Fellow of the National Cancer Institute. Present address: Physiology Dept., Tufts University Medical School, Massachusetts, U.S.A.

1. Schneider, W. C. and Hogeboom, G. H. *J. Biol. Chem.* **183** (1950) 123.
2. Shmukler, H. W. and Polis, B. D. *Abstracts Div. Biol. Chem. Am. Chem. Soc.*, Sept. 1953, p. 13c.
3. Polis, B. D. and Shmukler, H. W. *Ibid.* p. 72c.
4. Polis, B. D. and Shmukler, H. W. *Ibid.* p. 19c.