isolation of Na,K-ATPase is therefore necessary in order to understand the mechanisms of action of different regulators and, in particular, of ACh on enzymes.

The authors are grateful to Academician Yu. A. Ovchinnikov and to K. A. Dzhanzhugazyan, on the staff of the Institute of Bioorganic Chemistry, Academy of Sciences of the USSR, for interest and for providing the facilities for work with the purified preparation of Na,K-ATPase and serum, and also to Corresponding Member of the Academy of Sciences of the USSR T. M. Turpaev and to M. N. Semenova, on the staff of the N. K. Kol'tsov Institute of Developmental Biology, Academy of Sciences of the USSR, for advice and help with the work.

LITERATURE CITED

1. A. A. Boldyrev, Biokhimiya, 36, 826 (1971).
2. M. P. Danilenko, O. V. Esyrev, and A. M. Mustfin, Izv. Akad. Nauk Kaz. SSR, Ser. Biol., No. 5, 82 (1979).
3. K. N. Dzhanzhugazyan, N. N. Modyanok, and Yu. A. Ovchinnikov, Bioorg. Khim., 7, No. 12, 1970 (1981).
4. A. M. Mustafin, Usp. Sovrem. Biol., 82, No. 2, 276 (1976).
5. A. M. Mustafin, Usp. Sovrem. Biol., 87, No. 3, 373 (1979).
6. A. M. Mustafin and O. V. Esyrev, Byull. Éksp. Biol. Med., No. 8, 182 (1978).
7. H. Dlouha, J. Teisinger, and F. Vyskocil, Pflüg. Arch. Ges. Physiol., 380, 101 (1979).
8. I. L. Ellmann, K. D. Courtney, A. Valentino, et al., Biochem. Pharmacol., 7, 88 (1961).
9. V. A. Knight and H. W. Thomas, Life Sci., 18, 291 (1976).
10. W. B. Rathbun and M. V. Betlach, Anal. Biochem., 28, 436 (1969).

CHANGES IN SOME LIPID PEROXIDATION PARAMETERS OF ALBINO RAT LIVER MITOCHONDRIA DURING ANOXIC INJURY SIMULATED *IN VITRO*

V. I. Sorokovoi, Yu. M. Petrenko,
N. N. Mochenova, G. M. Nikitina,
and Yu. A. Vladimirov

UDC 616.36-008.922.1-008.64-07:616.47-008.949.16-49-093.6

KEY WORDS: anoxia; mitochondria; lipid peroxidation; antioxidative activity; chemiluminescence.

The results of experiments to study the role of lipid peroxidation (LPO) in anoxia now available can be divided into two groups. On the one hand, a decrease in antioxidant activity (AOA) is observed in lipid extracts from mitochondrial and microsomal fractions of various organs in ischemia [1, 2]. On the other hand, there is evidence of increased AOA in homogenates and mitochondrial fractions of organs surviving *in situ* [3, 4, 12]. Probably these two groups of experimental facts are not contradictory, but mutually complementary, for in one case the test object consisted of lipid extracts, whereas in the other it was organelles or homogenates.

The object of this investigation was to study the state of LPO in mitochondria during anoxia in a simpler system than the cell, namely mitochondria + Ca^{++} + anoxia *in vitro*, in which virtually all features of anoxic injury to organelles can be adequately simulated [11]. Changes in the kinetics of Fe^{++}-induced chemiluminescence (CHL) of mitochondria and liposomes from the total lipid fraction, the content of intermediate and end products of LPO, and also the effect of Mn^{++} and Cu^{++} on parameters of CHL in mitochondria, for they may be potential regulators of LPO in the cell [15], were investigated.

Laboratory of Experimental Cell Pathology, Institute of Human Morphology, Academy of Medical Sciences of the USSR, Moscow. Department of Biophysics, N. I. Pirogov Second Moscow Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR A. P. Avtsyn.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 543-546, November, 1984. Original article submitted March 16, 1984.

 0007-4888/84/9811-1492$08.50

EXPERIMENTAL METHOD

Mitochondria were isolated from albino rat liver by differential centrifugation in buffered 0.25 Msucrose medium with 2 mM EDTA between 600 g (10 min) and 8000 g (10 min) and recentrifuged in medium without EDTA at 8000 g for 10 min. Anoxic incubation of mitochondria (15-20 mg protein in 1 ml medium) was carried out in a plastic syringe with fine plastic needle under 0.1 mm in diameter in medium of the following composition: KCl 62.5 mM, sucrose 125 mM, $CaCl_2$ 30 μM, pH 7.4, at 22-24°C. O_2 consumption and reduction of NAD under these conditions took place in 2.5-4 min. Samples for determination of functional parameters and the level of free fatty acids (FFA), measuring 0.1 and 0.3 ml, respectively, were taken every 15 min. The polarographic investigations were carried out in a transparent plastic cell by means of a covered electrode (from "Radiometer," Sweden); the incubation medium contained sucrose 125 mM, KCl 62.5 mM, mitochondria 1-1.5 mg protein/ml, HEPES 2 mM, $MgCl_2$ 2 mM, and sodium succinate 10 mM or sodium malate + sodium glutamate, 5 mM of each, pH 7.4. In the course of the experiments the mitochondria passed through states 4^n-3-4° according to Chance [6], and ADP was added at the rate of 25-150-300 μM. The FFA content was determined by the method in [13] with additional treatment of the extract with silica-gel [14]. Mitochondrial lipids were extracted by the method of Bligh and Dyer [5]. Liposomes were formed from them and these were then frozen and thawed [9].

Fe^{++}-induced CHL was measured on a chemiluminometer of ordinary construction, using working concentrations of mitochondria of 0.4-0.5 mg protein/ml, and liposomes of 0.15 mg lipid/ml. To induce CHL, $FeSO_4$ was added to the cuvette up to a concentration of 0.1 mM. The end products of LPO were determined spectrophotometrically as absorption of the complex formed from them with 2-thiobarbituric acid (TBA), and intermediates were determined by the TBA test [8]. The results are presented in the form $M \pm \sigma$.

EXPERIMENTAL RESULTS

Under anoxic conditions, in the presence of 30 μM Ca^{++} in the incubation medium, hydrolysis of phospholipids and a parallel decrease in coefficients of acceleration and respiratory control due to a reduction in the rate of respiration in Chance's state 3 and an increase in state 4 for different respiration substrates. Total loss of respiratory control by the mitochondria was observed when the FFA level rose from 9 to 16-18 nmoles/mg protein usually by the 45th-60th minute of incubation. By that time the results of the TBA test showed an increase of 40% and the content of TBA-active product showed a tendency to increase.

The latent period (τ) and time of development of CHL (τ_{CHL}) of the mitochondria by the 120th minute of anoxia were approximately doubled, and the highest intensity of CHL (I_{CHL}) was observed at the 30th minute of anoxia (Fig. 1). Changes in the kinetics of CHL were similar to those observed in experiments on mitochondria isolated from the liver survivng *in situ*, and they indicate a shift in the ratio between pro-oxidant and antioxidant properties of the organelles in the course of anoxic injury, toward an increase in AOA.

In another series of experiments parameters of CHL were studied in liposomes obtained from mitochondrial lipids of intact and injured mitochondria (incubation time 1 h). A special technical feature of these experiments was that TBA-active products were recorded after CHL, and in that way the ability of the samples to undergo reoxidation was determined. The values of τ and τ_{CHL} of CHL for liposomes formed from lipids of injured mitochondria (Table 1) were smaller than in the control. Their absolute values, however, were greater than for mitochondria. Capacity for reoxidation also was greater in liposomes formed from injured mitochondria than in the control. These results are in good agreement with data in the literature [1, 2] and they are evidence that capacity for LPO is increased in lipid extract *in vitro* after anoxic injury to the organelles, and the content of antioxidant is reduced. It thus seems most likely that the increase in AOA of the mitochondria in anoxia is on account of antioxidants which are not extracted during extraction with a chloroform-methanol mixture.

In the series of experiments to study the effect of Mn^{++} and Cu^{++} on the parameters of mitochondrial CHL an increase in the concentration of these ions to 50 μM was accompanied by an increase in the values of τ and τ_{CHL}, whereas I_{CHL} fell by an order of magnitude or more. The effect was only slightly more marked in the case of mitochondria injured by anoxia (Fig. 2). Ability of the suspension of control and experimental mitochondria to carry out LPO, estimated from accumulation of TBA-active products, as the result of Fe^{++}-induced LPO likewise was reduced, if Cu^{++} was present in the incubation medium (Table 2); Mn^{++} and Cu^{++} were particularly effective on CHL in liposomes. In a concentration of 1 μM these cations sharply

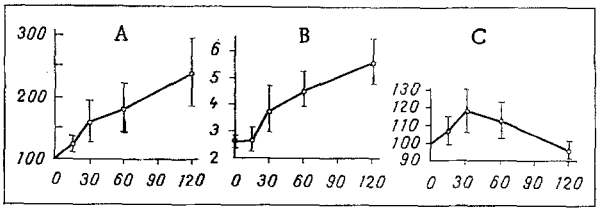

Fig. 1. Changes in latent period (A), period of development (B), and intensity (C) of slow flash of CHL of albino rat liver mitochondria during anoxia *in vitro*. Abscissa, duration of anoxic incubation (in min); ordinate: A) τ (in % of control), B) τ_{CHL} (in min), C) I_{CHL} (in %).

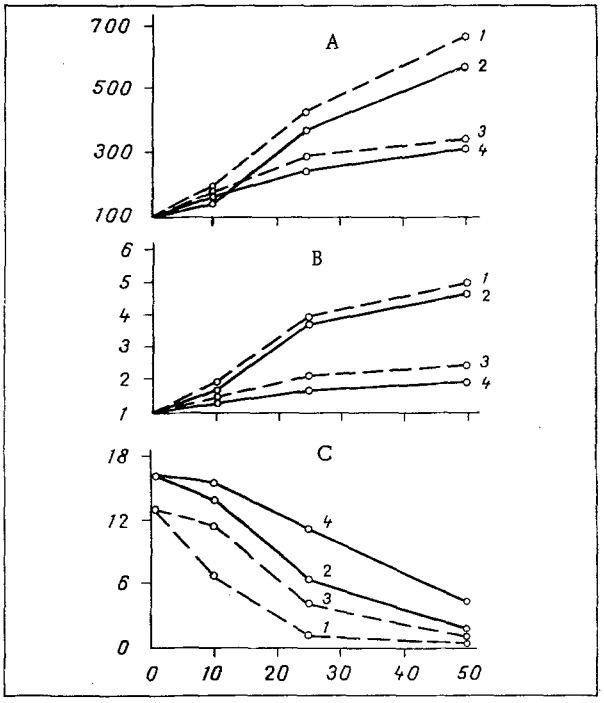

Fig. 2. Effect of Mn^{++} and Cu^{++} on latent period (A), period of development (B), and peak intensity of slow flash (C) of CHL of albino rat liver mitochondria. Abscissa, concentration (in μM); ordinate: A) τ (in % of control); B) τ_{CHL} (in min); C) I_{CHL} (in relative units). 1, 3) Anoxia (1 h); 2, 4) control. 1, 2) Cu^{++}; 3, 4) Mn^{++}.

TABLE 1. Parameters of CHL of Mitochondria and Liposomes Formed from Mitochondrial Lipids, and Their TBA-Active Product Level in Control and after 1 h of Anoxia *in vitro* (M ± σ)

Parameter tested	Mitochondria (n = 8)		Liposomes (n = 3)	
	control	anoxia	control	anoxia
τ, min	2,17±0,31	4,54±0,29*	8,34±0,17	5,63±0,34*
τ_{CHL}, min	3,26±0,37	6,32±0,17*	10,23±0,37	6,91±0,54*
I_{CHL}, %	100	113,5±10,2	100	193±22,8
TBA-active products, %	100±2,3	63,2±11,9*	100±3,9	117,8±7,0*

Legend. *P < 0.05 compared with control.

increased τ and τ_{CHL}, but the effectiveness of accumulation of TBA-active products in liposomes formed from injured mitochondria was reduced. In liposomes obtained from intact mitochondria CHL could not be recorded at all for 20-40 min.

TABLE 2. Dependence of Accumulation of TBA-Active Products After Fe^{++}-Induced LPO of Mitochondria on Mn^{++} and Cu^{++} Concentrations (M ± σ, n = 3)

Experimental conditions	TBA-active products, % of control, ion concentration, μM			
	0	10	25	50
Control + Cu^{2+}	100	83,8±3,2	71,8±4,3	59,3±5,8
Anoxia + Cu^{2+}	100	92,3±3,8	84,0±10,4	65,3±4,5
Anoxia + Mn^{2+}	100	94,3±4,9	89,3±14,0	74,7±21,5

Legend. In every case n = 3.

The results are evidence that Mn^{++} and Cu^{++} exhibit antioxidant properties in micromolar concentrations. No significant difference was found between the AOA exhibited by these cations in the case of intact or injured mitochondria. The most likely mechanism of action of these cations as antioxidants, from our point of view, is decomposition of hydroperoxide with the formation of inactive products. Regarding the possibility of involvement of Mn^{++} and Cu^{++} in the development of anoxic injury the following points may be noted: Although the concentrations of these cations in the cell cytosol is several orders of magnitude lower [15] than those which we tested, they can be carried into the cytosol from the plasma (where their concentration is measured in tens of micromoles [10]) during hypoxic injury to the cell *in vivo*, when the blood flow in the organ is partly preserved.

The results of the present investigation, in conjunction with the data in the literature, suggests that during anoxic injury to mitochondria *in vitro*, just as *in situ*, AOA of lipids of these organelles decreases whereas AOA of the mitochondria as a whole increases. In our opinion, either elevation of the level of thiol compounds in the matrix may be responsible for the increase in AOA, as noted in the experiments of Olenev et al. [7], or an increase in activity of enzyme systems producing inhibition of LPO may be responsible.

LITERATURE CITED

1. M. B. Bilenko, in: Bioantioxidants in the Regulation of Metabolism Under Normal and Pathological Conditions [in Russian], Moscow (1982), pp. 195-213.
2. E. B. Burlakova, A. V. Aleksenko, and E. M. Molochkina, in: Bioantioxidants in Radiation Lesions and Malignant Growth [in Russian], Moscow (1975), p. 214.
3. A. I. Zhuravlev, in: Bioantioxidants in the Regulation of Metabolism Under Normal and Pathological Conditions [in Russian], Moscow (1982), p. 187.
4. Kh. A. Kebedmagomedova, Yu. L. Mel'nikov, and Yu. A. Vladimirov, Biofizika, 15, 1022 (1970).
5. M. Kates, Techniques in Lipidology [Russian translation], Moscow (1975), pp. 72-77.
6. M. N. Kondrashova and Yu. V. Evtodienko, in: Handbook on the Study of Biological Oxidation by the Polarographic Method [in Russian], Moscow (1973), p. 93.
7. V. I. Olenev, "Investigation of the kinetics and regulation of lipid peroxidation in mitochondrial membranes," Candidate's Dissertation, Moscow (1976).
8. Yu. M. Petrenko, "Kinetics and mechanism of processes leading to disturbance of barrier functions of mitochondrial membranes during lipid peroxidation," Author's Abstract of Candidate's Dissertation, Minsk (1978).
9. V. I. Sorokovoi, A. V. Putvinskii, Yu. A. Vladimirov, et al., in: Compendium of Inventions and Efficiency Suggestions of Medical Schools and Research Institutes of the RSFSR [in Russian], Ivanovo (1974), pp. 223-225.
10. N. V. Semenov, Biochemical Components and Constants of Human Liquid Media and Tissues [in Russian], Moscow (1971).
11. V. I. Sorokovoi and Yu. A. Vladimirov, in: Biophysics [in Russian], Vol. 5, Moscow (1975), pp. 11-15.
12. V. I. Sorokovoi, Yu. M. Petrenko, and Yu. A. Vladimirov, Byull. Éksp. Biol. Med., No. 12, 113 (1983).
13. M. M. Anderson and R. E. McCarty, Anal. Biochem., 45, 260 (1972).
14. J. W. De Pierre, Anal. Biochem., 83, 82 (1977).
15. R. J. P. Williams, FEBS Lett., 140, 3 (1982).