ELECTRON-CYTOCHEMICAL INVESTIGATION OF THE BRAIN MITOCHONDRIA AT VARIOUS TIMES AFTER DEATH

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An electron-cytochemical investigation was made of oxidation of 3,3'-diaminobenzidine (DAB) in the brain of rats and man at different times after death. The oxidation product of DAB was localized in the mitochondria, lipofuscin granules, and erythrocytes. Oxidation of DAB by rat and human brain mitochondria was shown to be only very slightly depressed even 2 days after death.

KEY WORDS: postmortem changes; brain; 3,3'-diaminobenzidine; mitochondria.

Tissue taken at postmortem examinations is at the present time that which is most readily available for morphological investigation of nervous and mental diseases in man. The use of postmortem material for electron cytochemistry substantially broadens the scope of pathomorphology, but it raises the question of the suitability of material obtained at different times after death. From the moment of death the tissues of the body are under conditions of asphyxia, and this must probably be reflected in the functional and structural organization of the mitochondria. However, sections of nerve tissue in an oxygenated medium exhibited relatively high respiratory activity for several hours and the addition of exogenous substrates to the medium evoked metabolic responses similar in direction to those observed in mitochondria isolated from "fresh" tissue [4]. When postmortem material was used, mitochondria of nerve tissue were found to be in a good state of morphological preservation during the first 3 h after death; changes in metabolic activity of the mitochondria were reversible with respect to several indices. Under the influence of various agents the respiratory control of the mitochondria and coupling of oxidation with phosphorylation were fully restored 2 h after death, and the rate of oxidation was substantially increased [2]. When the neck muscles of an ox were kept for 144 h at 4°C the isolated mitochondria lost their orthodox configuration as it existed *in situ* and acquired a condensed configuration. Nevertheless, these mitochondria preserved 77, 85, and 65% of their initial ADP/O values, their respiratory control, and their rate of oxidation in state 3 in the presence of ascorbic acid and tetramethyl-N-phenylenediamine as substrates, respectively [7]. Mitochondria isolated from a pig's spinal muscle, kept for 48 h at 1°C, according to the spectrophotometric data, lost 47% of its cytochrome c (no fall in the levels of cytochromes aa_3 and c_1 was observed) and, if cytochrome c was added to the incubation medium, they were able to recover their initial level of respiration in state 3 to the extent of 80% [6]. The author cited suggests that the main factor in the reduction in the intensity of oxidation and phosphorylation after death is the loss of cytochrome c from the mitochondria.

With the above facts in mind, it was decided to investigate the localization of mitochondrial cytochromes in brain tissue *in situ* at various times after death.

EXPERIMENTAL METHOD

The frontal cortex and anterior hypothalamus from male albino rats was studied 0, 4, 8, 12, 24, and 48 h after death caused by acute blood loss (three animals were used at each time). The corresponding parts of the brain were investigated in human postmortem material (eight cases). This material was obtained 5, 8, 13, 15, 22, 26, and 46 h after death. In both cases

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the brain was kept *in situ* at room temperature (about 24°C) and was removed immediately before use. Pieces of tissue were minced in 0.08 M NaH_2PO_4–NaOH buffer, pH 7.4, to a particle size of the order of 300 μ, then transferred to an incubation medium made up, as described elsewhere [8, 9], but with certain modifications. Prefixation of the fragments in glutaraldehyde was not used, for it led to complete inhibition of the reaction in the mitochondria.

During preparation of the medium, 5 mg of 3,3'-diaminobenzidine (DAB; Sigma, USA) was ground in a drop of glycerol and the pH of the mixture was adjusted to 6.0 at 37°C with 0.1 M triethanolamine. The final volume of the incubation medium (2.50 ml) was obtained by the addition of 0.9% NaCl solution. The concentration of hydrogen peroxide in the medium was 0.04%. To inhibit the reaction of oxidation of DAB by mitochondrial cytochromes in the control experiments, inhibitors with an affinity for iron atoms in hemin molecules were used: KCN or NaN_3 in a final concentration of 10 mM. Pieces of brain tissues were incubated in the above medium with constant mixing for 1 h at 37°C. After incubation the pieces were quickly washed with physiological saline, fixed with 1% OsO_4 solution in 0.08 M NaH_2PO_4–NaOH buffer, pH 7.4, for 15 min at 4°C, and processed for electron microscopy [1]. Ultrathin sections of the tissue for examination were not stained additionally.

EXPERIMENTAL RESULTS

The electron-microscopic investigation showed that the reaction product can be observed in the same ultrastructures of both the rat's and human brain. Osmiophilic polymer of oxidized DAB was formed on the outer surface of the inner membrane of the mitochondria, filling the lumen of the cristae; it was also localized in lipofuscin granules and in erythrocytes. The use of the specific inhibitors KCN and NaN_3 completely suppressed the development of the reaction in the mitochondria, confirming the essential role of the cytochrome iron in the oxidation of DAB. The constant use of H_2O_2 in the medium stimulated the reaction, evidently through manifestation of the peroxidase activity of cytochrome c [3]. The use of the cytochemical reaction of oxidation of DAB by cytochromes thus can reveal changes in the localization of mitochondrial cytochromes in postmortem material. Comparison of the results obtained with rats and man showed that these changes were very slight in rats but appreciable in man. At the times indicated above, for instance, hardly any change in the localization of cytochromes was observed in the mitochondria of the rat's brain in observations on both the cortex and the hypothalamus (Fig. 1).

Not until 48 h after death was a diffuse distribution of the reaction product, or one with accompanying aggregate formation, observed in the matrix of isolated mitochondria of the corresponding brain formations. These observations agree with those of electron-microscopic studies of postmortem changes in the structure of mitochondria [5]. So far as the human brain was concerned, within the period of investigation after death the intensity of the reaction in the cortical and hypothalamic mitochondria fell a little. With an increase in the time of keeping of the cadaver, changes in the localization of the cytochromes under these circumstances were more severe in the mitochondria of the hypothalamus than in those of the cortex (Fig. 2). For instance, 5 h after death, the reaction product was not only localized in the cristae of many of the mitochondria of the human hypothalamus, but it was also diffusely distributed as aggregates in the mitochondrial matrix. Similar changes were observed in the mitochondria of the human frontal cortex only 26 h after death or later.

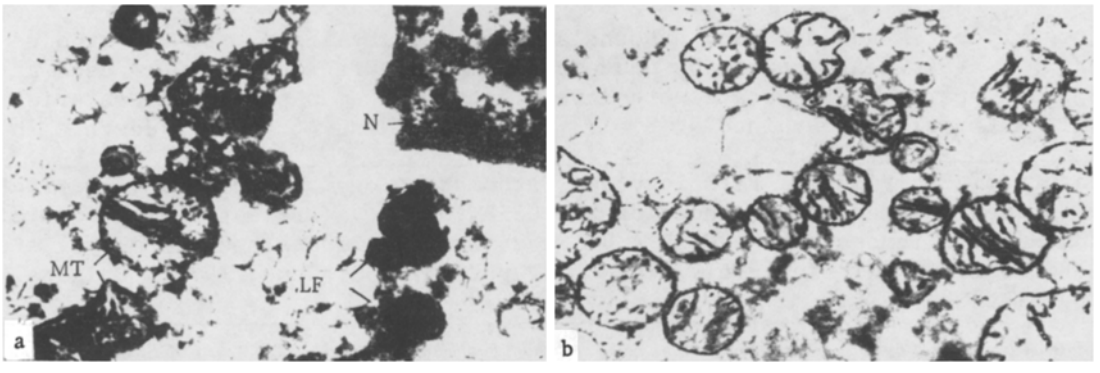

Fig. 1. State of ultrastructures of rat brain tissue 48 h after death: a) neuron of frontal cortex; MT) mitochondria; LF) lipofuscin; N) nucleus (24,000 ×); b) hypothalamic neuron (20,000 ×).

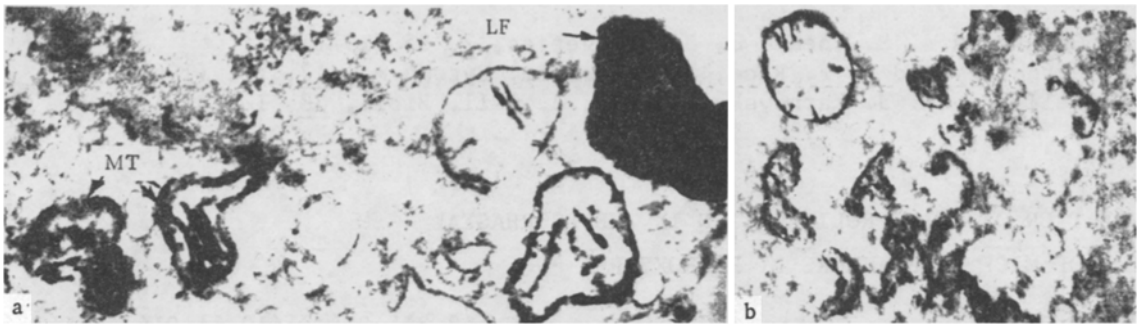

Fig. 2. State of ultrastructures of human brain tissue 46 h after death: a) neuron of frontal cortex: MT) mitochondria; LF) lipofuscin (30,000 ×); b) hypothalamic neuron (20,000 ×).

Since the reaction was carried out on unfixed material, incubation of fragments of rat and human brain led to some degree of destruction of part of the mitochondria, as shown by their deformation, rupture of the outer membrane, and destruction of the cristae and loss of their material outside the mitochondria. This led to the formation of the reaction product side by side with the disintegrated mitochondria. The essential fact was that in rats the number of destroyed mitochondria was very small in both cortex and hypothalamus and remained unchanged throughout the period from 0 to 48 h after death. Meanwhile in man the sensitivity of the mitochondria to the destructive action of the incubation medium increased the longer the cadaver was kept after death, and the number of destroyed mitochondria increased by a greater degree in the hypothalamus. The number of disintegrated mitochondria in human brain tissue investigated 5 h after death corresponded to that in rats at all times of the investigation. Naturally partial destruction of the mitochondria, attributable to the conditions of incubation, also provided some sort of indicator of the severity of their postmortem changes. However, a sufficient number of well-preserved mitochondria could always be observed at all times of the investigation, even 48 h (rat) and 46 h (man) after death. Moreover, all the changes observed affected the mitochondria of the cell bodies to a greater degree than mitochondria of the cell processes. The structure of the lipofuscin granules was unchanged after death.

To conclude, the more severe postmortem changes in the mitochondria in the human hypothalamus than in the cortex, and also than in the rat brain, can probably be explained by the slower decrease in temperature of the human hypothalamus after death. During the oxidation reaction of DAB by cytochromes in the tissue fragments no loss of cytochrome c from the mitochondria was observed, as had previously been reported in experiments *in vitro* on mitochondria isolated from cadaveric material [6].

This investigation demonstrates that human brain tissue can be used for the electron-cytochemical investigation of mitochondria as late as 2 days after death. The method based on oxidation of DAB can evidently be recommended not only for investigation of the morphology of the nervous system, but also for the study of the pathomorphology of poisoning connected with inhibition of the mitochondrial respiratory chain in occupational and forensic medical practice.

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LITERATURE CITED

1. V. A. Agafonov, Zh. Nevropat. Psikhiat., No. 7, 1083 (1975).
2. G. P. Gulidova, G. M. Maslova, Yu. I. Savulev, et al., in: Cell Respiration under Normal and Hypoxic Conditions (E. M. Khvatova, ed.) [in Russian], Gor'kii (1973), p. 53.
3. A. L. Kamyshnyi, E. S. Chukhrai, and O. M. Poltorak, Zh. Fiz. Khim., 48, No. 2, 334 (1974).
4. L. D. Luk'yanova, "Oxidative metabolism and reactivity of nerve tissue in surviving preparations and in the intact organism," Author's Abstract of Doctoral Dissertation, Moscow (1971).
5. E. F. Lushnikov and N. A. Shapiro, Autolysis. Morphology and Mechanisms of Development [in Russian], Moscow (1974).
6. K. S. Cheah, FEBS Letters, 19, 105 (1971).

7. K. S. Cheah and A. M. Cheah, J. Bioenergetics, 2, 85 (1971).
8. M. J. Karnovsky and D. F. Rice, J. Histochem. Cytochem., 17, 751 (1969).
9. A. M. Seligman, M. J. Karnovsky, et al., J. Cell. Biol., 38, 1 (1968).

SYNCHRONIZATION OF CELL PROLIFERATION IN THE ESOPHAGEAL EPITHELIUM OF MICE WITH TUMORS BY HYDROXYUREA

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The synchronizing action of hydroxyurea on the passage of esophageal epithelial cells through the S phase and mitosis was investigated in mice with tumors, making allowance for diurnal fluctuations in the number of these cells in particular phases under natural conditions. To evaluate synchronization *in vivo* two complementary criteria reflecting changes in the number of cells and the rate of change of synchronization are suggested. In artificial synchronization, two groups of cells passing synchronously through the S phase and mitosis were formed in the esophageal epithelium. The degree of artificial synchronization of the first group was less than natural. The number of cells in the second group in the period of DNA synthesis was twice the number of cells synthesizing DNA during natural synchronization, but the rate of change of synchronization was the same in the experimental and control groups.

KEY WORDS: index of labeled nuclei; mitotic index; synchronization; diurnal rhythm.

According to data in the literature, hydroxyurea, which synchronizes the passage of cells from the G_1 period into the S phase and DNA synthesis in experiments on animals *in vivo*, was sufficiently effective against both normal tissues [5, 8] and tumors [4, 7]. However, in the investigations cited no account was taken of diurnal rhythms of proliferative activity, the importance of which has been demonstrated several times when choosing the time of administration and analyzing the action of substances with an effect on particular phases of the mitotic cycle [1-3].

It was accordingly decided to investigate the synchronization of cell proliferation in the esophageal epithelium of mice inoculated with a tumor by means of hydroxyurea, allowing for diurnal fluctuations in mitotic activity and in the number of nuclei synthesizing DNA.

EXPERIMENTAL METHOD

Experiments were carried out on 120 male noninbred albino mice with a mean weight of 30 g. The animals were kept under natural conditions of illumination and with free access to food. Cell proliferation was studied in the basal layer of the epithelium of the lower third of the esophagus. For synchronization, the animals were given two intraperitoneal injections of hydroxyurea at 2 and 4 a.m., each in a dose of 100 mg/kg body weight. The compound was diluted before use in sterile isotonic sodium chloride solution. When given in this way, it was reckoned [6, 7] that the hydroxyurea would block the cells at the end of the G_1 period and in the S phase until 7-9 a.m.

On the second day after subcutaneous inoculation of all the mice with sarcoma 37, half of the animals (experimental group) were injected with hydroxyurea. The other half (control

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