PHOTOOXIDATION OF FLUORESCENT PREPARATIONS OF HUMAN BRAIN AUTOPSY MATERIAL FOR USE IN LIGHT AND ELECTRON MICROSCOPY

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Intracellular injection of Lucifer Yellow (LY) [2, 4, 8] and membrane transport of carbocyanin dyes [based on 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (dil)] [1, 7], are nowadays used for systematic detection of neurons and conducting tracts of the human brain. When relatively stable fluorescent dyes (LY and dil) are used, in the course of examination of the specimens the intensity of fluorescence fades, and there is also the risk of an increase in autofluorescence of preparations of this kind. Various methods of adapting fluorescent preparations for light (LM) and electron microscopy (EM) of the mammalian brain and biopsy material from the human brain have been described in the literature: methods involving the use of photooxidation of LY [4-6, 10-12] or dil [3] in a solution of 3,3'-diaminobenzidine-4HCl (DAB) and using antibodies to LY [8, 13].

The aim of this investigation was to study the possibility of using photooxidation with DAB in order to examine histological preparations of human brain autopsy material, stained by intracellular injection of LY or by membrane axonal transport of carbocyanin dyes.

EXPERIMENTAL METHOD

Human brain autopsy material (three cases: aged 61 years, diagnosis carcinoma of the esophagus; aged 65 years, pneumonia; aged 65 years, aortic aneurism), taken 7-16 h after death. LY was injected through a microelectrode into pyramidal neurons of the visual, frontal cortex, and nonpyramidal hippocampal neurons; carbocyanin dyes (based on dil) were injected into the white matter or into different layers of the visual cortex. A detailed description of these methods of injection of fluorescent substances into human brain autopsy material was given by the writer previously [1, 2]. Vibratome brain sections (thickness 75-150 μ), stained with LY or dil, were placed in a Petri dish and held firmly against its bottom by a metal weight; a solution consisting of 1 mg/ml DAB ("Poliscience") and 1 mg/ml of potassium cyanide ("Sigma," USA) in 0.1 M phosphate buffer (PB), pH 7.4, was added. For photooxidation of nerve cells stained with fluorescent dyes (precipitation of an electron-dense, dark-brown stable DAB reaction product at the location of the fluorescent substance) the Petri dish with section was placed under a "Zeiss ASM" fluorescent microscope (West Germany). Epifluorescence through objectives with magnification of 6.3 or 10 times and appropriate light filters were used: for LY with excitation maximum at 470 nm, for dil — 546 nm. After 1-2 h (the time varies depending on the density of illumination, the thickness of the preparation, and the fluorescent substance) the brain sections were washed 3 times with PB. Sometimes several photooxidation procedures were carried out on the same section, changing the DAB solution each time. For LM the sections were placed on slides with protein, dried, dehydrated in alcohols of increasing concentration, cleared in xylol, and mounted in balsam. For EM the sections were kept for 7-10 min in a 0.5% solution of osmium tetroxide ("Sigma," USA) in PB, washed 3 times with PB, dehydrated in alcohols of increasing concentration, and mounted between two cellophane leaflets in

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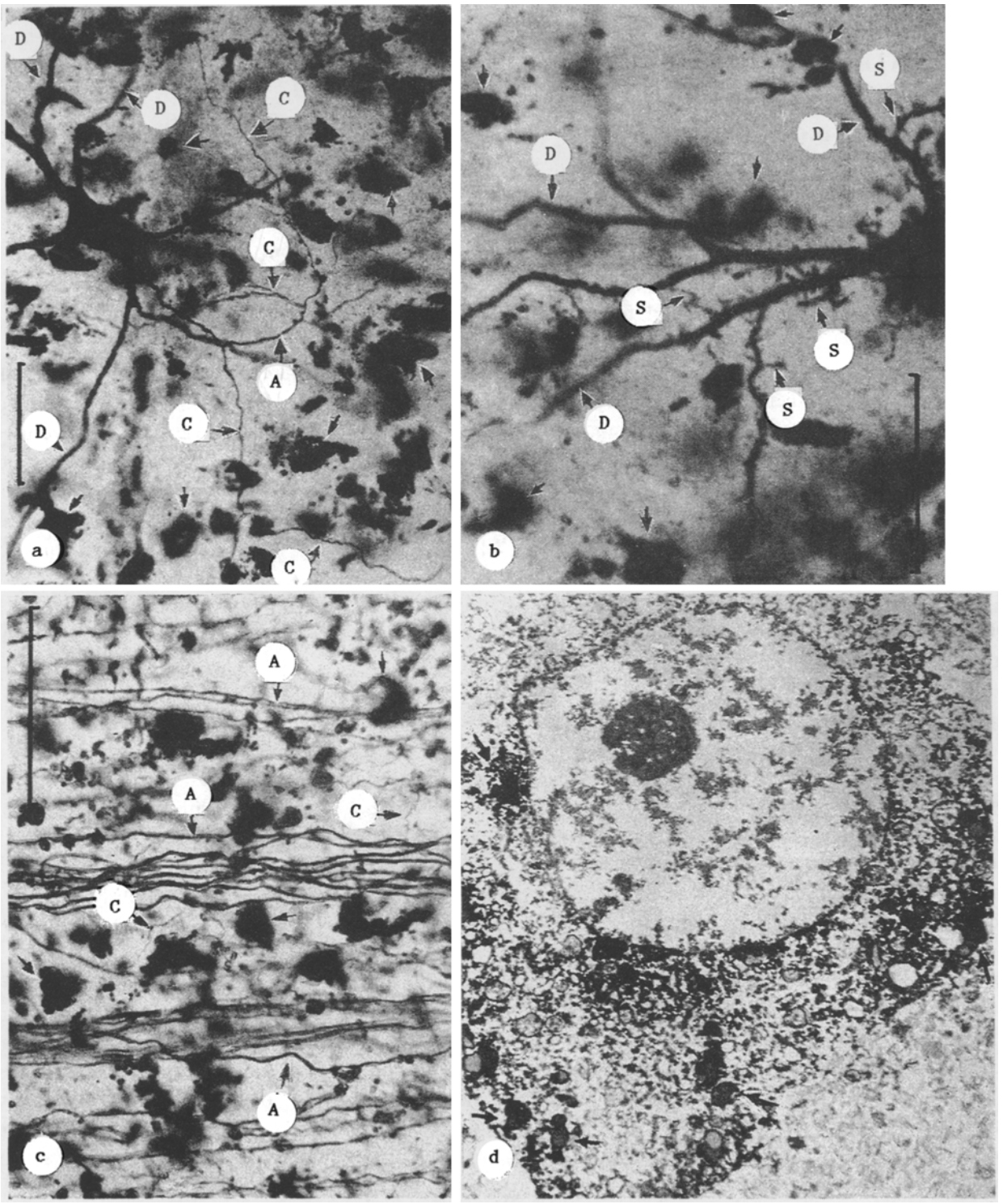

Fig. 1. Photooxidation of fluorescent preparations of human brain autopsy material: a) nonpyramidal neuron in area CA1 of the human hippocampus, stained by intracellular injection of LY and photooxidized; b) pyramidal neuron in hippocampal area CA1, stained by intracellular injection of LY followed by photooxidation; c) afferent fibers in human visual cortex, revealed by dil transport followed by photooxidation; d) EM of nonpyramidal neuron in hippocampal area CA1 after intracellular injection of LY and photooxidation. Arrows indicate lipofuscin granules. A) Axons, C) axon collaterals, D) dendrites, S) spines. Photomicrograph. For a, b, c, scale 50 μ; for d magnification 3300×.

"Durcupan" ("Fluka"). Serial sections 50 nm thick were obtained by means of a diamond blade on an ultramicrotome, and were mounted on grids coated with Formvar. Sections stained with LY were counterstained with uranyl acetate. Sections stained with dil were not so treated, for in this case it is impossible to distinguish at the electron microscopic level membranes stained with dil and membranes stained with uranyl acetate [3]. Preparations for LM were analyzed under the "Zeiss ASM" microscope and those for EM under the "Zeiss EM-10" microscope ("Leitz," West Germany).

EXPERIMENTAL RESULTS

The use of photooxidation of fluorescent substances is a quite easy and reliable method of obtaining histological preparations for LM and EM. Analysis of histological preparations of human brain autopsy material, photooxidized after intracellular injection of LY, under the light microscope showed that the neurons were stained together with all their dendrites, dendritic spines, axons, and their collaterals (Fig. 1a, b). Photooxidation of human brain autopsy material after injection of the carbocyanin dye dil revealed at the LM level bundles of axons with collaterals and with beads along their course (Fig. 1c); single terminal fibers with beads along their course or with spinelike formations were very clearly stained; neurons also were demonstrated with all their details. Such preparations are very convenient for making morphometric calculations of the parameters of neurons and fibers. On electron micrographs, a neuron stained by intracellular injection of LY, and then photooxidized, has a darker outline of its cytoplasm (Fig. 1d): dendrites also have a darker profile. However, the fine cytological structure of these elements is difficult to study because of the DAB reaction product. Since in the human adult the pigment lipofuscin, which has autofluorescence, accumulates in the neurons, glia, and vessels, after photooxidation its granules also are visible during LM (Fig. 1a, b, c) and EM (Fig. 1d), but this does not interfere with analysis of the preparations.

By using photooxidation by our modified method [11] for experimental morphological investigations of the human brain the fluorescent preparations can be converted into light microscopic preparations which are also suitable for EM. Preservation of autopsy material consisting of nerve tissue for EM is mainly dependent on the time the material was taken after death, and it is completely unaffected by the process of injection of the fluorescent dyes on their photooxidation. It has been found that tissue for EM is better preserved in human brain biopsy material [4, 5] and also after staining with the carbocyanin dye dil in material fixed for 2 years [3].

Photooxidation followed by LM is particularly valuable for preparations stained with dil, for it is the only method allowing a detailed and long-term study of nerve cell morphology, without having to worry about fading of the fluorescence, which is a feature of traditional fluorescence microscopy [1, 7]. As regards the use of photooxidation for preparations stained with LY, this technique is much easier than the method using antibodies to LY, which is used to examine them under the light microscope [8, 13], most of all because of poor penetration of antibodies into very thick preparations. Staining single neurons with LY greatly simplifies the process of comparing light-optical data with ultrastructure, and also the obtaining of morphological data. There is also the possibility of undertaking immunomorphological studies of preparations stained with dil for both LM [9] and EM [3].

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LITERATURE CITED

1. P. V. Belichenko, Byull. Éksp. Biol. Med., No. 5, 500 (1990).
2. P. V. Belichenko, Arkh. Anat., No. 4 (1991).
3. C. S. Bartheld, D. E. Cunningham, and E. W. Rubel, J. Histochem. Cytochem., **38**, No. 5, 725 (1990).
4. E. H. Buhl and W. Schlote, Acta Neuropath. (Berlin), **75**, 140 (1987).
5. E. H. Buhl and J. Lubke, Neuroscience, **28**, No. 1, 3 (1989).
6. E. H. Buhl, W. K. Schwerdfeger, P. Germroth, and W. Singer, J. Neurosci. Meth., **29**, 241 (1989).
7. A. Burkhalter and K. L. Bernardo, Proc. Nat. Acad. Sci. USA, **86**, 1071 (1989).
8. G. Einstein, J. Neurosci. Meth., **26**, 95 (1988).
9. A. J. Elberger and M. G. Honig, J. Histochem. Cytochem., **38**, No. 5, 735 (1990).

10. P. Germroth, W. K. Schwerdtfeger, and E. H. Buhl, Brain Res., **494**, 187 (1989).
11. A. Maranto, Science, **217**, 953 (1982).
12. J. H. Sandell and R. H. Masland, J. Histochem. Cytochem., **36**, No. 5, 555 (1988).
13. F. G. Wouterlood, B. Jorritsma-Bynam, and P. H. Goede, J. Neurosci. Meth., **33**, 207 (1990).

CELL ULTRASTRUCTURE IN THE PYGMY SHREW

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In connection with the study of the fine structure of rat hepatocytes engaged in intensive physical exercise (running) under experimental conditions [4] it is interesting to compare data thus obtained with ultrastructure of the hepatocytes of animals which normally are characterized by high motor activity. Pygmy shrews are small animals feeding on insects, their larvae, earthworms and seeds [1]. In their search for food they are on the move for a large part of the day, and consume two to four times as much food as their body weight [1, 8]. It has been shown experimentally that the interval between feeding is 15 min for the common shrew and 10 min for the pygmy shrew [6].

EXPERIMENTAL METHOD

The ultrastructure of the liver cells of juvenile female pygmy shrews (*Sorex minutissimus*), the lesser shrew (*Sorex minutus*), and a juvenile female common shrew (*Sorex araneus*), caught in July in the Yenisei taiga (Mirnyi settlement), was studied. Pieces of liver were fixed in a 2.5% solution of glutaraldehyde in S-collidine buffer, pH 7.2-7.4, and post fixed with OsO_4. The material was embedded in Epson and sections, stained by Reynolds' method, were examined in the JEM-100C electron microscope. The relative volumes of the organelles and inclusions were studied by means of a random-step grid [7]. Since the ultrastructure of the liver cells of the three species of shrew studied is basically similar, we give a description and the results of counting done on the hepatocytes of the pygmy shrew. For instance, the relative volume of mitochondria in the liver of the pygmy shrew was 35.6 ± 0.20 conventional unit, of the rough endoplasmic reticulum 6.35 ± 0.06, the smooth endoplasmic reticulum 4.05 ± 0.03, the Golgi complex 4.25 ± 0.04, secondary lysosomes 1.35 ± 0.03, with autophagosome 0.4 ± 0.05, peroxisomes 2.8 ± 0.03, and glycogen 8.75 ± 0.14.

Both mono- and binuclear hepatocytes were seen. The nuclear membrane formed numerous pores. There was little heterochromatin and it was localized along the nuclear membrane. A quite large nucleolus lay in the karyoplasm. The numerous round and oval mitochondria with moderately dense matrix were conspicuous (Fig. 1). Very many cristae were present in the matrix, and many mitochondria were dividing along the cristae. Each mitochondrion was surrounded by a small, flat cistern of the rough endoplasmic reticulum (RER). A few vesicular profiles of the smooth endoplasmic reticulum (SER) were located in the cytoplasm. The Golgi complex was small and consisted of one or two flat cisterns and tiny vesicles. Secondary lysosomes with finely granular contents were seen in the zone of the Golgi complex. Peroxisomes were quite numerous, measured 0.4-0.6, and did not contain a nucleoid. The biliary capillaries were closed. Many ribosomes were present. There was no fat in the cytoplasm, and small concentrations of glycogen were not found in every hepatocyte. Small autophagous vacuoles containing mitochondria or myelin figures were seen.

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