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LIPID EXTRACTION FROM ISOLATED SINGLE NERVE CELLS

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Translation of "Ekstratsiya lipidov iz otdel'nykh izo-
lirovannykh nervnykh kletok," Tsitologiya, Vol. 15,
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16. Abstract A method of extracting lipids from single neurons isolated from lyophilized tissue is described. The method permits the simultaneous extraction of lipids from 30-40 nerve cells and for each cell provides equal conditions of solvent removal at the conclusion of extraction.					
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LIPID EXTRACTION FROM ISOLATED SINGLE NERVE CELLS

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Quantitative fluorometric analysis of enzyme activity in /1535 isolated single nerve cells opens up bright methodological possibilities for quantitative cytochemistry of the nervous system (Lowry et al., 1956; Lowry, 1957; Robins and Hirsch, 1968; Hirsch, 1970). Yet the interpretation of data obtained by this method in studies of the brain neurons of animals subjected to a variety of physical and chemical factors (hypercapnia, hyper- and hypoxia, modified gravity, pharmacological agents and so on) is quite complex since the enzyme activity in a single nerve cell is calculated with respect to the weight of its dry tissue--weight of protein, lipids, and RNA of the cell. Since the lipid content in a nerve cell, 30-50 percent of the weight of the neuronal body, can change by nearly 50 percent when exposed to extremal factors (Brattgard and Hyden, 1954), it becomes obvious that conducting quantitative analysis of the activity of enzymes in a single neuron without determining the weight of the "defatted" dry neuronal tissue after the lipids have been extracted appreciably hampers evaluating the results. This present study proposes a method of extracting lipids from single neurons isolated from lyophilized tissue that permits simultaneous extraction of lipids from 30-40 nerve cells and that ensures for all cells equal conditions of solvent removal after extraction.

Analytical Procedure and Results

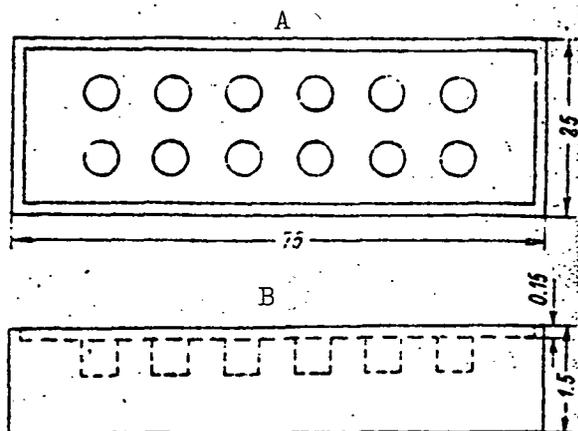
Extractor. Lipids were extracted from nerve cells in an extractor: a slide made with rows of depressions--"wells" in

* Numbers in the margin indicate pagination in the foreign text.

the center of the slide and rounded at the perimeter with a glass shoulder 0.15 mm in height (see Figure). The "wells"-- 1.5 mm in diameter and 1 mm in depth--are arranged in the slide in two to three parallel rows running lengthwise along the slide, 17 "wells" in each row. In each row the "wells" are 2-2.5 mm apart. The rows are 3 mm apart. On the obverse side of the slide, numbers are scribed opposite each "well" for identification. The extractor is covered with a second slide as a cover and placed in a mandrel, stainless steel, 0.4 mm thick. At the bottom of the mandrel is a square opening, 13x68 mm, permitting the extractor "wells" to be viewed in transmitted light.

Making and cleaning the extractor. Drops of India ink are placed on the slide at the locations of the future "wells"; after the drops have dried, the slide is coated with a thin layer of paraffin and with a specimen-preparing needle round openings 0.5 mm in diameter are made in the paraffin over the beads of ink. Then using an ocular scalpel, the ink is removed from the slide surface at the location of the openings in the paraffin and the slide is immersed for 2 h in hydrofluoric acid. The action of the acid forms depressions of the required size in the glass. The shoulders of the extractor are made of strips of cover glass 0.15 mm thick and are secured to the slide with the "wells" with epoxy resin. After repeated use of the extractor, the "wells" are periodically washed by filling them with "chromic mixture," then rinsing the extractor with water and drying it in vacuum at $5 \cdot 10^{-2}$ mm Hg. /1536

Extraction procedure. Placement of nerve cells in "wells." The latter are filled with solvent and the cells are extracted from the "wells" after extraction, under inspection with an MBS-2 stereomicroscope at magnification 16 to 32. Before the cells are placed in the "wells" the extractor cover is shifted and each neuron (after preliminary determination of its weight



Extractor for extracting lipids from isolated single nerve cells

A. Top view B. Side view

Dimensions are in millimeters

on a quartz-filament scale) /1537 using a quartz or a hair needle 10 mm thick is placed in a separate "well," corresponding to its number. Therefore, during the subsequent manipulations with the neuron, its identification does not present any difficulties. Using a glass pipet with a 100 μ tip diameter, the "wells" containing the cells are successively filled with solvent. To prevent premature evaporation of the solvent, the "wells" are covered with the second slide that is pushed over them as they are filled.

After the "wells" have been filled, the extractor--kept horizontal--is rolled into a 16-18 μ aluminum foil and in order to evaporate the solvent at 20 $^{\circ}$, the extractor is placed horizontally in a glass ampule used to lyophilize tissue sections (Krasnov, 1967). From the ampule, with an RVN-20 vacuum pump monitored with a VIT-1A vacuum meter, air is evacuated to a $5 \cdot 10^{-2}$ mm Hg vacuum. Then the ampule is disconnected from the vacuum system, the extractor is taken out of the ampule and placed under the stereomicroscope. The weight of the cells after lipid extraction is determined again on the quartz-filament scale. The lipid weight is calculated from the difference between the weight of the cells before and after lipid extraction.

Lipid Content in Motor Neurons of the Anterior
Horns of the Cervical Level of the Spinal Cord
of the Monkey Macaca rhesus

a № нейрона	b Вес нейрона (в мг)		e Относительное содержание липидов в сухой ткани (в %)	f Вес экстра- гируемых липидов (в мг)	g Относительное содержание экстрагируемых липидов (в %)
	c до экстрак- ции	d после экстракции			
1	12.22	9.10	74.50	3.12	25.50
2	9.95	7.01	70.40	2.95	29.60
3	6.22	4.55	70.00	1.87	30.00
4	6.83	4.17	61.40	2.63	38.60
5	9.77	5.85	69.00	3.91	40.00
6	7.76	5.15	65.50	2.61	33.50
7	6.30	4.41	70.00	1.89	30.00
8	10.07	6.34	61.70	3.93	38.30
9	9.10	5.84	64.20	3.26	35.80
10	5.13	3.35	65.30	1.78	34.70
11	6.55	4.27	65.20	2.28	34.80
12	4.48	2.84	68.40	1.64	36.60
13	5.50	3.33	60.80	2.17	39.40
14	7.39	5.17	70.00	2.22	30.00
15	10.54	6.68	64.10	2.71	35.90
16	6.63	4.55	65.80	2.31	34.70
17	3.91	2.40	61.40	1.51	35.80
18	6.42	4.55	68.00	2.06	32.00
19	6.65	4.35	65.20	2.31	34.70
$\bar{x} \pm s_x$			65.65 \pm 0.84		34.35 \pm 0.94
r_{11}			3.82		-3.98
Коэфф. вари- ации			6.02%		11.60%
t			69.80		36.60

Remark. Lipid extractions was conducted simultaneously
from all neurons.

Key: a. Neuron number b. Neuron weight (in mg)
c. before extraction d. after extraction
e. Relative content f. Weight of extracted lipids
of "defatted" dry (in mg).
tissue (in percent) g. Relative content of ex-
tracted lipids (in per-
cent)
h. Coefficient of
variation

We employed successive extraction of cells with absolute ethanol for 3 min to remove the the lipids from the lyophilized neurons, followed by two extractions--3 min each--with heptane. After each extraction, the solvent is removed from the extractor in a vacuum. The weight of the cells is determined on the quartz-filament scale; its sensitivity is 17 ng/mm. The table gives the results of determining the weight of motor neurons of the anterior horns of the cervical level of the spinal cord of the Macaca rhesus monkey before and after lipid extraction using the method proposed above. The relative content of "defatted" dry tissue and lipids in the bodies of the motor neurons is 65.65 and 34.35 percent, respectively.

Discussion

Fluorometric determination of enzyme activity in isolated single nerve cells, based on the fluorometry of pyridine nucleotides (Lowry et al., 1956; Lowry, 1957) or 4-methylumbelliferone (Robins and Hirsch, 1968; Hirsch, 1969, 1970; Hirsch and Obenchain, 1970), includes as an essential stage in the analytical procedure determining the weight of dry tissue on a quartz-filament scale. The enzyme activity found by cytochemical analysis of the enzyme can be expressed in moles of the fluorophore formed, calculated per unit weight of dry tissue per unit time (per kg of dry tissue per h). Calculation of the enzyme activity with respect to the weight of the dry tissue--even though widely used in quantitative chemical analysis of separate neurons isolated from lyophilized tissue--can hardly be regarded as well-grounded owing to the relatively high lipid content in the cytoplasm of nerve cells. In the cytoplasm of

the neurons of Deiters' nuclei in rabbits, lipids are 50 percent of the weight of the dry tissue, in the case of motor neurons of the spinal cord--26-50 percent, and for the Purkinje cells in the cerebellum--22 percent; here the relative lipid content in the cytoplasm as a result of changes in the functional state of the neuron is subjected to significant fluctuations, changing in turn the relative content of the carrier of enzymatic properties --protein (Brattgard and Hyden, 1954). Evidently, referring to the latter Lowry (Lowry, 1953, 1957) proposed extracted lipids from single neurons and calculating the enzyme activity with respect to the weight of the "defatted" dry tissue. In extracting the lipids, the neuron is placed in the bottom of a micro test tube and is treated successively with absolute ethanol and hexane. The weight of the cell before and after extraction is determined on a quartz-filament scale. However, in the attempt to reproduce this procedure, we were unable to get satisfactory results, since the attempt often proved unsuccessful owing to the impossibility of extracting the nerve cell from the micro test tube after extraction, because the cell adhered to the glass surface. Also, an equal degree of solvent evaporation could not be achieved after extraction for each cell, within the limits of the neuron population analyzed; and the solvent remaining in the tissue, together with air moisture condensing on the tissue during its evaporation, could add to cell weight differently for each cell. The instability of the results and the laboriousness of the procedure makes it virtually inapplicable for the simultaneous analysis of 30-40 neurons--the number that is the minimum cell population for statistical evaluation. Evidently, methodological difficulties in extracting liquids can account for the fact that the study by Lowry (Lowry, 1957) is the only one where the enzyme activity in single neurons was expressed with respect to the weight of the "defatted" dry tissue. All authors of later

studies conducted on neurons isolated from lyophilized tissue expressed enzyme activity only with respect to the weight of dry tissue.

The method of extracting lipids from single neurons isolated from lyophilized tissues proposed in this study is simple, technically speaking; it permits simultaneous extraction of lipids from 30-40 nerve cells--a number sufficient for the statistical analysis of the cell population, and ensures for all cells equal conditions of solvent removal at the completion of extraction. Adhesion of cells on the glass surface of the "wells" was not observed. The coefficient of variation, when this method served in determining the relative content of "defatted" dry tissues in the motor neurons of the anterior horns of the cervical level of 153 the spinal cord of the monkey, was only 6 percent. However, it rose to 16 percent in the analysis of the giant neurons of Deiters' nuclei in the monkey, when the relative content of the "defatted" dry tissue in these cells was 64 percent.

Lowry (Lowry, 1953, 1957) extracted lipids from single neurons with absolute ethanol and then twice, with hexane, each time for 3 min. This procedure fully removes lipids from a specimen of lyophilized nerve tissue 20x15x40 μ in size. When the relative content of lipids in the pyramidal layer of the hippocampus containing only neuronal bodies was analyzed, Lowry (Lowry et al., 1954) secured identical results when lipids were extracted with hexane and chloroform. The relative lipid content in the cytoplasm of rabbit motor neurons, found by histoentgenography in single lyophilized cells after chloroform extraction of lipids and in the histological sections of the spinal cord after fixation with Karun's fixative--containing absolute ethanol and chloroform, was the same (Brattgard and Hyden, 1954). In extracting lipids with heptane and hexane, we arrived at the same results;

however, the first solvent was to be preferred owing to its higher boiling point (98°) and thus the lower rate of evaporation from the "wells."

The test of completion of lipid extraction from the cell was the absence of weight change after the next extraction. A repeated extraction with heptane after extraction with absolute ethanol is quite sufficient to establish the stable neuron weight.

Besides lipids, RNA can be a source of error in calculating the enzymatic activity in single cells with respect to the weight of the dry or "defatted" tissue. The relative weight content of RNA in the dry tissue of nerve cells is 1 percent in motor neurons of the rat spinal cord (Ford and Cohan, 1968; Geynisman et al., 1971) and about 7 percent in the neurons of the hypoglossal nerve cells (Brattgard et al., 1957) and in Deisters' nuclei (Hyden, 1959) of the rabbit. After lipids were removed from dry tissue, the relative RNA content in the neurons of Deisters' nuclei rose to 10 percent, and it would appear the change in the RNA content in the neuron in differential functional neuronal states may have been a source of substantial errors in the calculating the enzyme activity with respect to the weight of "defatted" dry tissue. However, it should be stressed that the enzyme activity in the cell body, 40-60 μ in diameter and isolated from a 15-20 μ thick lyophilized section, is determined not entirely in the neuronal body, but only in part of it (part of the cytoplasm) and in calculating the enzyme activity use is made only of the weight of part of the cell (part of the cytoplasm); so here it is not the change in the absolute amount of RNA in the cell that is important, but the change in its concentration with respect to the weight of the "defatted" dry tissue (weight concentration). All the experimental factors studied thus far as acting on the animal organism and its pathological states change the RNA concentration in the cytoplasm of neuronal bodies independently of

which particular brain structure they originate in by not more than 30 percent of the initial level taken as normal. The only exception is the regeneration of the axon after dissection (Brattgard et al., 1957; Edstrom, 1959). The change in the RNA concentration in these limits is equivalent to a change in the relative content of cytoplasmic RNA by 3 percent with respect to the weight of the dry cytoplasm; therefore, in calculating the enzyme activity with respect to the weight of the "defatted" dry tissue, the error growing out of the possible change in the amount of RNA in the cytoplasm will not be substantial. Doubtless, more exact results can be achieved by a replicate determination of the RNA content in the neuronal cytoplasm, with the appropriate correction made in the weight of the "defatted" dry tissue.

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