THE QUANTITATIVE HISTOCHEMISTRY OF SUPRAMEDULLARY NEURONS OF PUFFER FISHES

I. Enzymes of Glucose Metabolism

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Current investigations in our laboratory have been focused on problems of physiological control mechanisms of the neuron as studied with chemical methods. These studies are based on the concept that some of the more important keys to the understanding of nervous system function and malfunction must be sought at the cellular level. Consequently, several technological approaches have been developed which can both generate hypotheses and permit investigation at this level. The microspectrophotometric methods of Caspersson have been applied to the neuron by Hydén and his collaborators (6), and the elegant technology of the Cartesian diver respirometer of Linderstrom-Lang and Holter has been brought to the single cell level by the ampulla diver of Zeuthen (2–8). However, these techniques are applicable only to fixed tissue or cells isolated from the living brain and maintained under relatively unphysiological conditions. Therefore, the functional capacity and state of the cell are necessarily indeterminate to some degree. An ideal technique should permit the study of single neurons or cytologically homogeneous regions of the nervous system in a known functional state. The methods of Lowry appear to offer the closest approach to such an ideal. Rapid freezing should arrest function under known conditions, and drying without thawing should prevent subsequent changes which might be expected to occur due to persistent enzyme action or diffusion of soluble components during the isolation of single cells and their analysis. Microanalysis of cell components and metabolic intermediates can be undertaken after destroying all enzyme activity, or the activities of specific enzymes can be analyzed at high dilution of tissue so that endogenous substrates have little effect on activity (13). Moreover, a large number of different determinations can be carried out on tissue from the same experimental subject. Thus, one may obtain simultaneous data and internal controls that are uninfluenced by the common sources of variance arising in experiments by the use of different individual animals at different times and frequently under different conditions. Our methods are essentially extensions of the basic Lowry techniques.

The supramedullary neurons of the puffer fishes are particularly well suited for this approach because of the large size of their perikarya (200–400 μ in diameter) and their superficial location on the dorsal medulla which is covered only by a thin layer of glia and arachnoid (1, 14). Their location permits physiological manipulation such as stimulation and recording as well as extremely rapid freezing (within 2 sec), thus arresting chemical events in a known functional state.

The present paper essentially represents a validation of the methods used in physiological studies. The methods should yield an accurate picture of conditions existing within the cells immediately prior to freezing when functional parameters have been clearly defined. Such information can hardly be obtained by other means at this time. The data to be presented also represent a contribution to knowledge of the enzymology of the cell nucleus.

MATERIAL AND METHODS

Adult specimens of *Diodon hystrix* and *Spheroides spengleri* were immobilized, and respiration was maintained by circulating aerated seawater through their gills. The medulla was exposed and frozen *in situ* by the direct application of a stream of Freon-12, chilled to its freezing point (−150°C) by liquid nitrogen. With the stream of Freon still running, the frozen medulla was rapidly removed and transferred in liquid nitrogen to a −80°C deep freeze unit for storage until further handling. Transverse sections were cut on a calibrated microtome at 20 μ in a cryostat at −25°C and dried, without thawing, under

vacuum at less than −45°C. The details of tissue handling and dissection methods have been described (9, 13, 15). Single supramedullary neurons were dissected from each section. Only such cells were used in which the section passed through the equator of the cell. This was determined by observing that the cell as well as the nucleus subtended the same area on both sides of the section. The cells were trimmed to remove invaginating capillaries (14); in some cases fragments of nuclei and cytoplasm were separately dissected. As an assurance of the accurate measurement of volume, special care was taken in dissecting so that all cuts were made perpendicular to the plane of the section. The cell fragments, weighing from 0.01 to 0.04 μg, were weighed on a quartz fiber balance and subsequently photographed through a low-power microscope. A hemocytometer grid was photographed at the same magnification. The photographs were enlarged without changing the enlarger setting. Silhouettes of the cell fragments and reference squares were cut from the dried prints and weighed. This procedure allows extremely accurate determinations of areas of the sections with less than 0.2% standard error of the mean. The areas were then multiplied by the section thickness of 20 μ, and this value was used as a measure of the tissue volume. The dimensional stability of tissues on freezing and drying under the conditions used here is better than 1%. The accuracy of this volume measurement, therefore, is well within the limits of the experimental error of the over-all methods used (less than 10% standard error of the mean). The tissue fragments were weighed on a quartz fiber fishpole balance which gave a displacement of 1.00 mm for 19.0 ng (10^{-9} g). The methods and conditions for the enzyme measurements have been described (10). All analyses were made on 6–8-fold replicates from the same specimen. In these measurements the cell fragments weighed from 10 to 40 ng, and the incubation volumes were between 1 and 2 μl. Final fluorometer readings were made on a 50 μl volume in special, flat-bottomed tubes with a 4 mm inside diameter. As a check on the over-all enzyme procedures and to control possible day-to-day variability, homogeneous samples of rat cerebellum granularis were run with every determination.[1] For interspecies comparison, fish cerebellum granularis was also analyzed. These samples were also dissected from freeze-dried sections under direct microscopic control.

RESULTS

In *Spheroides* relatively little difficulty was encountered, and operative procedures were well tolerated; but an unexpected problem was encountered in the larger *Diodons*. The subarachnoid space normally is filled with a viscous gel through which course the blood vessels that supply the meninges. Attempts to remove this material by suction led to considerable bleeding and subsequent damage to the underlying brain. The material could be removed, however, by applying a dilute solution of hyaluronidase.

The density of *Diodon* supramedullary neuron cell bodies was 0.182 ± 0.008 kg of dry weight per liter of cell volume; the corresponding density for isolated nuclei was 0.132 ± 0.007 and for cytoplasm, 0.196 ± 0.018.

Results of determinations for hexokinase (HK)[2] lactic dehydrogenase (LDH), malic dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G-6-PDH), and β-D-glucuronidase (GLR) are given in Table I. All results are expressd as moles of substrate converted per kilogram of tissue dry weight per hour (MKH). Raw data for fragments of nucleus and cytoplasm are given in Table II for two enzymes, G-6-PDH and HK. In most cases, the standard errors of the mean were small, and there was excellent agreement among replicate determinations. β-glucuronidase was found only in the cytoplasm, as would be expected of a lysosomal enzyme. The distribution of this enzyme in the samples represents a check on the accuracy of the dissection methods and assures that none of the nuclear samples are contaminated with cytoplasmic components. All other enzymes studied were present in both nucleus and cytoplasm, LDH and MDH at approximately equal levels and HK and G-6-PDH twice as high in cytoplasm as in nuclei. Both LDH and MDH were relatively low in the perikarya of supramedullary neurons, and G-6-PDH was relatively high as compared to the cerebellum granularis of the same fish. The fish cerebellum granularis was remarkably close in enzyme levels to that of the rat. When recalculated on the basis of density measurements, the differences between nucleus and cytoplasm became somewhat, but not greatly,

[1] Lehrer, G. M., M. B. Bornstein, C. Lichtman, and C. Weiss. Carbohydrate metabolism of the developing rat cerebellum *in vivo* and *in vitro*. In press.

[2] The following abbreviations were used in this text: G-6-PDH, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, 1.1.1.-49); GLR, β-glucuronidase (β-D-glucuronide glucuronohydrolase, 3.2.1.31); HK, hexokinase (ATP: D-hexose 6-phosphotransferase, 2.7.1.1); LDH, lactate dehydrogenase (L-lactate: NAD oxidoreductase, 1.1.1.27); MDH, malic dehydrogenase (L-malate: NAD oxidoreductase, 1.1.1.37).

TABLE I

Enzymes in Single Fish Supramedullary Neuron Cell Bodies

	HK	LDH	MDH	G-6-PDH	β-D-GLR
	MKH	*MKH*	*MKH*	*MKH*	*mMKH*
Diodon					
Nuclei	1.77	5.20	64.5	3.87	0*
	(0.10)	(0.47)	(2.4)	(0.20)	
Cytoplasm	3.51	5.20	71.1	7.20	68.2
	(0.25)	(0.40)	(2.0)	(0.28)	(9.7)
Whole cell bodies (slices)	2.58	7.00	80.8	5.05	48.3
	(0.09)	(0.71)	(3.1)	(0.54)	(5.7)
Cerebellum granularis	3.14	54.5	197.0	2.05	36.5
	(0.18)	(1.2)	(7.6)	(0.09)	(4.5)
Spheroides					
Whole cell bodies	1.87	26.9	102.0	1.68	79.0
	(0.20)	(2.0)	(7.0)	(0.81)	(5.8)
Rat					
Cerebellum granularis	4.36	62.6	318.0	1.69	26.6
	(0.34)	(0.8)	(7.4)	(0.06)	(0.5)

Figures in parenthesis are standard errors of mean.
* Not significantly different from blank values.

TABLE II

Raw Data for Enzyme Determinations on Nuclear and Cytoplasmic Fragments of Single Diodon Supra-Medullary Neuron Perikarya to Show the Reproducibility of the Methods

	Dry wt.	G-6-PDH	Dry wt.	HK
	10^{-2} μg	*MKH*	10^{-2} μg	*MKH*
Nuclei	2.54	4.49	1.84	1.33
	1.21	3.59	2.51	1.82
	1.50	4.47	3.84	1.61
	1.61	3.41	2.01	1.89
	2.00	3.53	4.08	2.12
	1.48	3.72	2.26	1.84
Cytoplasm	6.88	5.84	2.64	3.08
	6.40	6.34	3.38	2.52
	4.05	7.16	3.04	4.07
	4.58	7.54	2.66	3.14
	2.54	7.42	3.08	3.45
	2.54	7.38	2.01	4.17
	3.42	8.34		
	4.56	7.58		

accentuated. For example, when hexokinase was recalculated on the basis of moles of substrate converted per liter of cell water per hour, the values were 0.197 for *Diodon* nuclei and 0.510 for cytoplasm.

DISCUSSION

Since all the enzyme assays were carried out at 37°C, values for the fish and rat may be directly compared. Values for enzymes in the granularis in the fish cerebellum were very close to those of rat cerebellum, except for the value of MDH which was slightly lower in the fish than in the rat. Levels of G-6-PDH in *Diodon* supramedullary neuron cell bodies were relatively high, those of HK and MDH were somewhat lower, and those for LDH were extremely low. However, values for HK and MDH in *Diodon* were extremely close to those for single neurons cell bodies in rabbit dorsal root ganglion cells as reported by Lowry (12, 13). By comparison, G-6-PDH activity was considerably higher in the fish neurons, and LDH activity was extremely low. A possible explanation for the low values for LDH and MDH in the puffer supramedullary neuron perikarya may lie in the extremely rich capillary blood supply of these cells. Each cell is surrounded by a glomus of capillaries which invaginate the outer cytoplasm of the cell and are separated from it only by a basement membrane and an extremely thin layer of glial processes (14). Thus, these cells may have a much richer oxygen supply than most other neurons. Consequently, if relative anoxia were the stimulus for synthesis of lactic dehydrogenase, it is conceivable that conditions would be such that the

production of LDH would not be stimulated as intensely nor as frequently in these cells. Lowry also found MDH to be relatively lower in ganglion cell bodies than in whole brain or *stratum radiatum* of Ammon's horn. Here again, the enrichment of oxygen supply, by increasing efficiency of oxidation of pyridine nucleotides via the electron transport system, may shift steady-state conditions in favor of oxidation, thereby increasing flux through the tricarboxylic acid cycle and thus decreasing the requirement for enzymes. Such a concept can be tested by determining the relative state of oxidation of pyridine nucleotides. Experiments with this aim are in progress and will be reported in a later paper.

Our findings regarding the distribution of enzymes between nucleus and cytoplasm are in remarkably close agreement with those of Lowry for dorsal root ganglion cells of the rabbit. For example, where Lowry finds a ratio for HK between cytoplasm and nucleus of 1.98 (13), in our data this ratio is 1.94; similarly, Lowry's ratio for MDH is 1.14 (12) and ours is 1.10. Since we were unable to find any evidence of GLR activity in our nuclei, we feel confident that our nuclear samples were not contaminated by cytoplasmic enzyme. Siebert using an anhydrous cell fractionation technique, found similar nuclear-to-cytoplasmic ratios for LDH, MDH, and G-6-PDH in rat liver, pig kidney, and beef brain (16). However, his separation of nuclear material from cytoplasm was not epecially clean. The reader should note that it has not been possible to demonstrate any of the soluble pyridine nucleotide-dependent enzymes in nuclei by any of the slide-histochemical methods (11). This should emphasize the fact that such methods are far from reliable, particularly in the sense that the absence of a localized product conveys no information about the presence or absence of the enzyme.

SUMMARY

Single supramedullary neuron cell bodies and fragments of nuclei and cytoplasm of these cells were dissected from freeze-dried sections of the brain of *Diodon hystrix* and *Spheroides spengleri* and analysed for a number of enzymes. There was good agreement among replicate determinations. β-glucuronidase was found only in the cytoplasm. Lactic dehydrogenase and malic dehydrogenase were found at approximately equal levels in both the nucleus and cytoplasm, whereas hexokinase and glucose-6-phosphate dehydrogenase were present in the nucleus at approximately one-half the activity found in the cytoplasm. Lactic dehydrogenase was remarkably low in *Diodon* supramedullary neurons, and glucose-6-phosphate dehydrogenase was relatively high as compared with mammalian single neurons and other sites in the fish nervous system; otherwise, enzyme levels were comparable.

Support for this work was provided by Public Health Service grant No. NB-05368 from the National Institute of Neurological Diseases and Blindness, grant No. 293 from the National Multiple Sclerosis Society and grant No. 62 from the Damon Runyon Foundation.

Received for publication 12 December 1967.

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