Sodium and Potassium Distribution in Puffer Fish Supramedullary Nerve Cell Bodies

ROBERT KATZMAN, GERARD M. LEHRER, and CLARENCE E. WILSON

From the Saul R. Korey Department of Neurology, Albert Einstein College of Medicine, Bronx, New York 10461, and the Department of Neurology, Mt. Sinai School of Medicine, New York 10029

ABSTRACT The Na and K concentration in single supramedullary neurons of the puffer fish (*Spheroides maculatus*) was measured using a dual channel integrating ultramicroflame photometer. The cells were frozen *in situ*, sectioned at low temperatures, and freeze-dried to prevent artefactual movements of cations. The density of the nuclear fragments was 0.15, significantly less than cytoplasm's 0.21. The sucrose-^{14}C "space" was 2.1–4.7% in cytoplasm fragments and 0.9–2.1% in nuclear fragments. The K concentration in cytoplasm averaged 134 mmoles/liter tissue volume and in nuclei, 113. The Na concentration in cytoplasm fragments varied between 56 and 138 mmoles/liter per tissue volume; in nuclei between 40 and 135, and in perineural tissue between 55 and 114. This intracellular Na is several times greater than the Na concentration expected from previous estimates. It is probable, however, that the intracellular Na activity is less than half that of the Na concentration, suggesting that much of the intracellular Na is bound to organic molecules within the cell.

INTRODUCTION

In this paper we report on the Na and K concentration of single supramedullary neurons of the puffer fish (*Spheroides maculatus*) and of cytoplasmic and nuclear fragments from these cells. Our interest in the Na and K concentration of these neurons is based upon the difficulty in assigning the high Na content of vertebrate nervous tissue. Under the Hodgkin-Huxley-Keynes formulation, Na is considered to be chiefly extracellular. Eccles (1957) has estimated that in the cat motoneuron, the intracellular Na is about 15 mmoles/kg and the extracellular about 150. However, in many species the total brain Na varies between 40 and 80 mmoles/kg. To assign these high levels of Na to extracellular space might, in the most extreme condition, require an extracellular space close to 50% of the tissue water. This is clearly

incompatible with electron micrographic studies of the cortex, and even assuming that there is some dehydration leading to an artificially small extracellular space on electron micrographs, the largest estimates from use of the so-called tracers are of the order of 20 %. Thus, it seems likely that in many instances the intracellular concentration of Na in the brain is quite large. Katzman (1961) postulated that glia might be high Na cells, accounting for these results. However, Kuffler and Nicholls (1966) demonstrated that, in fact, astroglia are high K cells whose membranes act as almost perfect K electrodes. The possibility, therefore, remains that the intraneuronal Na may be rather large. This is confirmed by the present study. The extent to which this finding is compatible with the Hodgkin-Huxley-Keynes account of the generation of the nerve impulse in these cells will be discussed.

The supramedullary neurons of the Atlantic puffer fish (*S. maculatus*) offer an unique opportunity for such a study of intracellular electrolytes in vertebrate neurons. These cells lie in a cluster of 30 or 35 cells on the dorsal surface of the brain stem and cord behind the cerebellum. The cells are very large, with diameters of 200–300 μ in some specimens. The fine structure of these neurons has been studied by Nakajima et al. (1965). The electrophysiology of these neurons has been reported in detail by Bennett et al. in a series of papers (1959 *a*, *b*, *c*; 1967).

METHODS

Since the supramedullary neurons lie on the dorsal surface of the medulla and cord, they are readily accessible for quick freezing. In the experiments to be described, this portion of the nervous system was exposed and irrigated with artificial CSF containing sucrose-^{14}C or inulin-^{3}H to define the extracellular space. After allowing the sucrose or inulin to equilibrate, the irrigating solution was removed, and the tissue frozen *in situ* with Freon-liquid nitrogen. Subsequently, the tissue was kept at low temperatures to prevent movement of cations. Na and K analyses were carried out on dissected freeze-dried specimens using a dual channel integrating ultramicroflame photometer.

In most experiments, adult specimens of *S. maculatus* were used, although several determinations were done on *Diodon hystrix* and *Spheroides spengleri*. The *S. maculatus*, netted near Sandy Hook, New Jersey,[1] were 16–22 cm long, weighing 100–200 g. *D. hystrix* and *S. spengleri* were obtained off the Florida coast. The specimens were first maintained in seawater and then transferred to aquaria containing a mixture of seawater and artificial seawater (Instant Ocean). The aquaria were vigorously oxygenated. The animals were processed between 14 and 96 hr after netting. The experimental method for handling of the animals has been previously described (Bennett et al., 1959 *a*; Lehrer et al., 1968). The respiration of the immobilized fish was

[1] The authors are grateful to Mr. Dave Duell and Mr. W. Mahoney of the Marine Biological Laboratory, Sandy Hook, N. J., and Dr. Ross Nigrelli and Mr. Robert Morris of the New York Aquarium.

maintained by perfusing the gills with a jet of oxygenated artificial seawater. After exposure of the supramedullary neurons, this area was irrigated with artificial CSF (Na, 150 moles; K, 3; Ca, 1.3; HCO_3, 22; PO_4, 1.5; Mg, 1.0; Cl, 130, and SO_4, 1.0) containing sucrose-^{14}C or inulin-^3H. The specific activity of the sucrose-^{14}C was 420 mc/mmole, and the final activity was 1 mc/ml of CSF. The specific activity of the inulin-^3H was 25 mc/g, and the activity was 2 mc/ml of CSF. The irrigating fluid was sampled and replaced after 10 min. In most experiments, the total time of irrigation was 30 min, but in some animals the time was as short as 15 or as long as 90

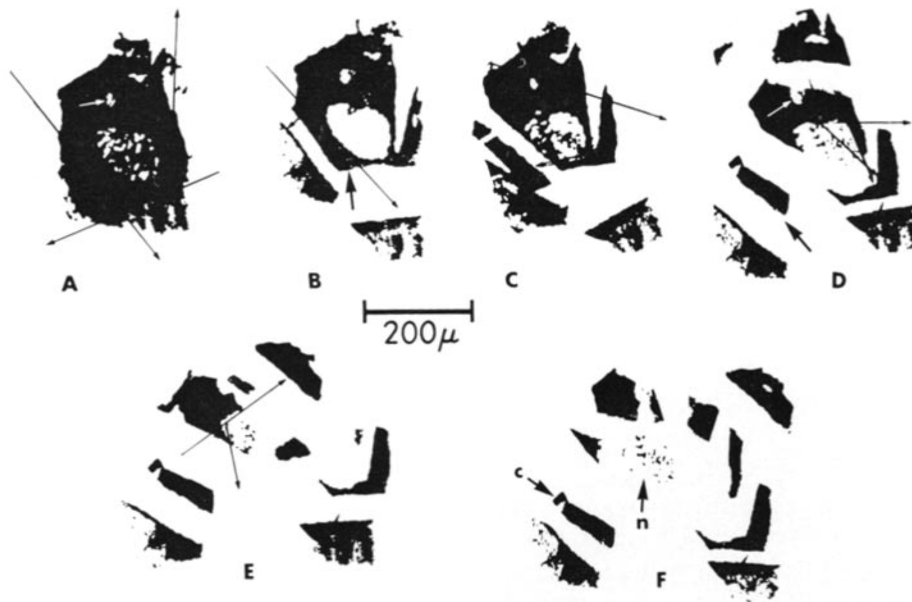

FIGURE 1. Progressive stages in the microdissection of a single equatorial 30 μ section through a puffer fish supramedullary nerve cell body. The horizontal marker corresponds to 200 μ. These pictures of dry, unstained tissue were taken by transmitted light, and the denser areas of cytoplasm appear black. The less dense nucleus is lighter. The slender arrows indicate cuts to be made, which have been executed in the next frame. The white arrow indicates an invaginating capillary, which is easily identified and which is to be avoided. The examples of nucleus and cytoplasm which are to be analyzed are labeled *n* and *c* in F.

min. At the end of the experiment, the irrigating fluid was blotted off, and the exposed tissue was frozen *in situ* using Freon-12 cooled to $-150°C$ with liquid nitrogen. The frozen tissue was then kept at dry ice temperature. The tissue was processed using the technique of Lowry (1953). This method as applied to the supramedullary neurons of puffer fish has been described (Lehrer et al., 1968). Transverse sections were cut on a calibrated microtome at 30 μ in a cryostat at $-25°C$ and dried, without thawing, under vacuum at less than $-45°C$. Single supramedullary neurons were dissected under microscopic control from each section. Only those 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. Special care was taken that all cuts were made perpendicular to the plane of the section. The cells were then trimmed to remove invaginating capillaries as shown in Fig. 1. The capillaries can be easily seen in the dissecting microscope. However, in the dissections, the outer third of the cell, where the endocellular capillaries are found, was trimmed away, and fragments of cytoplasm nearest the nucleus as well as individual nuclei were separately dissected. The cell fragments, weighing from 0.01–0.04 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

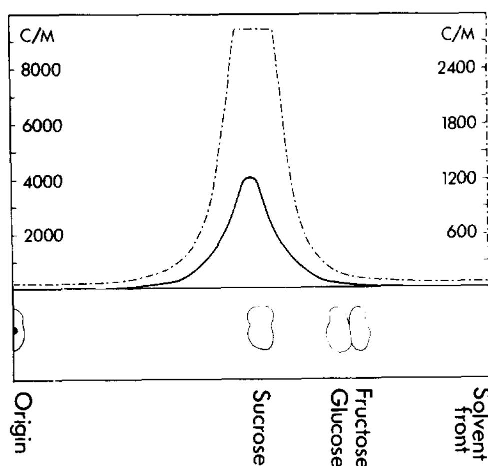

FIGURE 2. A radioisotope scan of a chromatograph. A TCA extract of cerebellum and cord, which had been exposed to sucrose-^{14}C for 30 min, has been chromatographed on Whatman 3 MM paper using formic acid:tertiary butanol:water 15:70:15 (v/v/v) as the solvent. Nonradiographic sucrose, glucose, fructose, glucose-6-phosphate, and lactate were added as carriers. The lactate and glucose-6-phosphate remain at origin. A 4 π scan was carried out: The solid black line corresponds to the scan at low gain, and the dotted line is a scan of the same strip at high gain. It can be seen that all counts correspond to the sucrose spot.

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% SEM. The areas were then multiplied by the section thickness of 30 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 described 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. However, a further measure of the reliability of this method was provided by a series of gelatin standards prepared with the sucrose-^{14}C. These were frozen, lyophilized, sectioned, and dissected in the same manner as the tissue. The volume of the sections was determined and the sections were counted. There was good correlation

between the volume estimated from the photographs and the volume as determined by the sucrose content.[2]

The radioactivity of the ^{14}C specimens was determined by counting on planchettes in a gas flow counter with a background of less than 0.5 cpm. The counts were at a rate of about 10 times background for the least active specimens. Each specimen was counted to 2,000 counts twice in order to reduce the probable error of the counting statistic.

No enzyme is known in vertebrate brain capable of breaking down sucrose. To check the chemical integrity of the sucrose-^{14}C, sections of cerebellum and cord adjacent to the supramedullary neurons were extracted with TCA and chromatographed on Whatman 3 MM paper using formic acid:tertiary butanol:water, 15:70:15 (v/v/v) as the solvent. Nonradioactive sucrose, glucose, fructose, glucose-6-phosphate, and lactate were added as carriers. The chromatographic strips were then scanned for radioactivity. All the counts were in the sucrose spot as shown in Fig. 2.

In order to assess the state of oxygenation of the tissue, lactic acid and phospho-

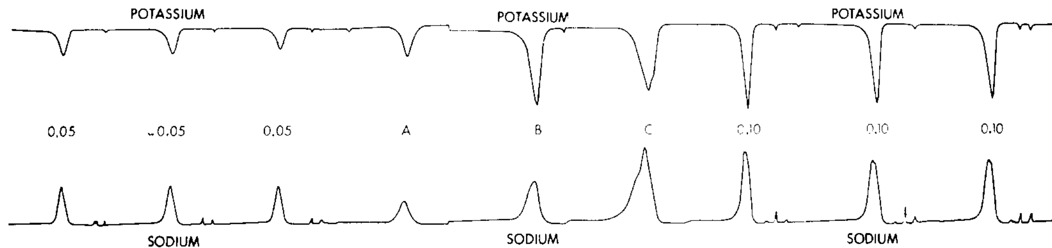

FIGURE 3. A typical Visicord writeout of the dual channel integrating ultramicroflame photometer. The vertical deflections correspond to the voltage output of the flame photometer during the burning of each specimen. The standards contain an equimolar mixture of Na and K: The three on the left consist of 0.24 λ of 0.05 mEq/liter of NaCl and KCl, respectively. The three on the right consist of 0.24 λ of 0.10 mEq/liter of NaCl and KCl, respectively.

creatine content were determined on the cryostat sections using methodology previously described (Maker et al., 1966).

The Na and K content of macroscopic portions of puffer fish brain was measured by atomic absorption spectroscopy. Sections of the puffer fish brains were cut at −20°C in a moist chamber so that the frozen tissue could be weighed immediately. Portions with a fresh weight of 3.4–7.5 mg were then analyzed.

The Na and K content of cytoplasmic and nuclear fragments of single supramedullary neurons and of the surrounding perineural tissue was measured by the use of a dual channel integrating ultramicroflame photometer. Our instrument is similar in concept and design to those of Ramsey et al. (1953), Bott (1960), Oberg et al. (1967), Müller (1958), and Carlsson et al. (1967). The principle of these instruments is simple. The light emitted by excitation of the Na or K in a flame is resolved into the individual components using interference filters and detected by a photoelectric

[2] Lehrer, G. M., R. Katzman, and C. E. Wilson. 1969. Volume and density measurements in subcellular portions of single nerve cell bodies. *J. Histochem. Cytochem.* In press.

cell as in an ordinary flame photometer. However, the light emitted throughout the 2 sec period of excitation is recorded and integrated. Our instrument employs a small oxygen-methane ("high purity" methane, Matheson, East Rutherford, N. J.) flame, whose light is focused by a lens system through the interference filters onto the photoelectric cells (General Electric photovoltaic cell 8PB). The voltage output of the photo cells is electronically amplified and simultaneously displayed on an oscilloscope and written out on a Honeywell Visicorder (Honeywell Co., Minneapolis, Minn.). The emission peaks are both integrated electronically, and the Visicorder output is integrated mechanically. The interference filter selected to pass the Na line has an extremely narrow bandwidth, transmitting light at 595 ± 11 mμ. Since the intensity of the K emission is much less than that of Na, a wider band filter was selected, 755 ±

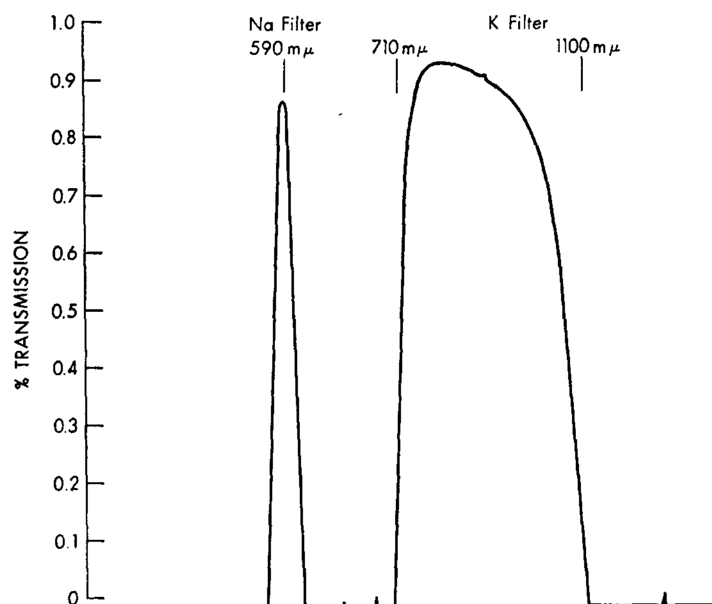

FIGURE 4. Transmission spectra of Na and K filters. The filters are measured by the Perkin-Elmer 350 recording spectrophotometer. The per cent transmission is plotted against wavelength. The Na filter has an extremely narrow bandwidth of only 22 mμ, whereas the K filter transmits light between 710 and 1100 mμ. The separation of Na and K filters is about 110 mμ.

195 mμ. The transmission spectra of these two filters are shown in Fig. 3. It can be seen that despite the bandwidth of the K filter, there is no overlap between the Na and K filter, that is, there is no response to a Na sample on the K channel, and vice versa (Fig. 4). The sample delivery system consisted of a platinum-iridium (85:15) wire mounted on a motor-driven rotating platform. By the use of microswitches, the rotating platform could be stopped either with the wire in the center of the flame or at a position 90° from this, suitable for applying the sample. In order to ensure an accurate stop so that the wire would be centered in the flame on each occasion, a beveled disc brake was used to slow the rotating platform just prior to the tripping of the micro-

switch. The platinum-iridium wire was formed into a ½–1 mm loop at its end. The loop is designed to hold 0.24–1.0 λ of fluid (quartz-redistilled water or a standard). The dried specimens were added to the water on the loop. It was found that the prior addition of water to the loop prevented loss of sample material and ensured more uniform burning. Although this photometer is sensitive to less than 10^{-12} moles of Na and K, this degree of sensitivity was not required in the present experiments in which the actual Na content varied between 10^{-9} and 10^{-11} moles. The typical Visicord record for both standards and tissue samples is shown in Fig. 5. Typical standard curves for Na and K are shown in Fig. 6. The excellent linearity of Na in the range of 10^{-11} moles can be seen. It should also be noted that occasional values deviate from the standard curve, introducing in these instances an error in the Na concentration of 5–10%. These occasional erratic determinations occurred because of random fluctuations in flame in-

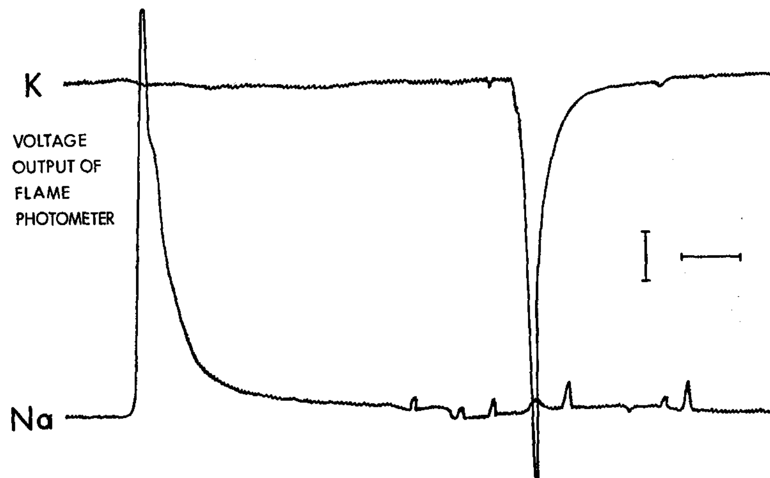

FIGURE 5. Visicord writeout on both Na and K channels of single cation. The two channels were at equal gain. The vertical marker corresponds to a 25 mv output of the flame photometer. The horizontal time marker equals 1 sec. The peak on the left was a response to 0.24 λ of 0.15 mEq/liter of NaCl, and the response on the right was to the same units of KCl. No crossover of Na or K is seen.

tensity. Fewer fluctuations occur with the K, but the standard curve for K tends to flatten out at higher concentrations.

In order to determine the effectiveness of the system in estimating Na and K in the presence of tissue constituents, 7.4 mg of wet weight of kitten cortex was homogenized in 10 ml of distilled water. The Na and K were determined in aliquots of this homogenate with and without the addition of a standard which contained 1.2×10^{-11} moles of Na and K. In triplicate determinations, 92.9% of the added Na and 96.4% of the added K were recovered. In order to test the reliability of the entire process of freeze-drying, dissection, volume estimation, and cation analysis, 11.2 g of gelatin were dissolved in 100 ml containing 25 meq/liter of Na and 100 meq/liter of K. Since the gelatin contained 99 mg % NaCl, final Na and K concentrations were: 26.9 meq/liter Na and 100 meq/liter K. A small capsule containing this gelatin solution was then frozen in liquid Freon and processed in the same manner as the

frozen tissue. The volume of dissected fragments was determined photographically, and the Na and K measured on the dual channel integrating ultramicroflame photometer with the following results:

Volume	Na	K
10^{-9} liter	*meq/liter*	*meq/liter*
0.517	32.5	102.6
0.351	29.9	105.2
0.522	28.7	107.1
0.520	28.8	105.6

On the basis of these data, it would seem reasonable to assume that the usual error in our over-all method is less than 10%.

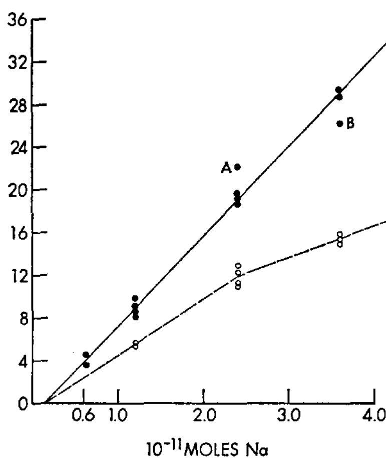

FIGURE 6. Standard curves for Na (solid line) and K (broken line) on the integrating ultramicroflame photometer. The units on the ordinate represent the integrated output, that is, the area under the curve as seen in the examples in Fig. 3. The units represent cm² in the Visicord writeout. Linearity of Na writeout is better than for K, but erratic values A and B are more often seen on the Na channel.

RESULTS

Na and K of Puffer Fish Brain

The Na and K content of puffer fish brain is shown in Table I. The K concentration of puffer fish brains varied between a value of 92 mM in *D. hystrix* and 129 mM in *S. maculatus*. These values for brain K are well within the limits seen in other vertebrate species. The Na content of the brain in these fish, however, is surprisingly high, ranging from 65 mM in *D. hystrix* to 83 mM in *S. maculatus*. In mammals, the Na content varies between 45 mM in rats and

60 mM in man (Van Harreveld, 1966).[3] In mammals, the Na and K content is higher in brain than in most other tissues (Katzman, 1961). Moreover, in mammals the Na content apparently increases as the brain becomes more complex. In puffer fish, however, with a relatively simple brain, the Na content exceeds that found in human brains.

The high tissue Na raises the possibility that brain edema may have been present. It is conceivable that netting and captivity in an aquarium might lead to a pathological response of the brain with a subsequent increase in Na content. There is no apparent evidence of anoxia. The appearance of the medulla and cerebellum at the time of operation was not edematous nor did ordinary histology show edema. The animals studied by Nakajima et al. (1965) and Bennett et al. (1959 *a, b, c*) were handled in a fashion similar to the present one. In these fish, the ultrastructure did not indicate overt edema, neither extra- nor intracellular, nor did the electrophysiological studies suggest

TABLE I

Na AND K CONTENT OF PUFFER FISH BRAINS. ANALYSIS BY ATOMIC ABSORPTION SPECTROSCOPY

Fish	N	Na	K
Spheroides maculatus	6	83.1±7.7	128.8±9.6
Medulla and cerebellum			
Spheroides spengleri			
Optic lobe	4	78.2±4.2	96.6±7.1
Cerebellum	4	71.4±6.9	94.7±8.0
Cord	4	79.4±5.2	99.9±5.9
Diodon	2	65.2	91.8
Cerebellum and cord			

that the cells had been damaged. Thus, there is no evidence suggesting cerebral edema, but the possibility has not been rigorously excluded.

Density

The method employed has permitted a direct determination of the density of nuclear and cytoplasmic fragments as shown in Tables II and III. The nuclear fragments had an average density of 0.15, whereas the cytoplasmic fragments had an average density between 0.21 and 0.23. This difference is highly significant whether one compares the data from individual fish or from the entire set of nuclear and cytoplasmic fragments.

Intracellular Sucrose Space

The sucrose spaces in cytoplasmic, nuclear, and perineuronal fragments are shown in Tables II–IV. The sucrose space of 7.5–10.3 % observed in the

[3] Katzman, R., G. M. Lehrer, and C. E. Wilson. 1969. Possible intraneuronal location of an "extracellular" tracer ^{14}C sucrose. *J. Neuropathol. Exp. Neurol.* In press.

perineuronal fragments is close to the values obtained in mammalian brain tissue when the surface of the brain is perfused with sucrose. An interesting finding is the presence of a 3 % sucrose space in cytoplasmic fragments. We

TABLE II

PUFFER FISH. CYTOPLASMIC FRAGMENTS OF SUPRAMEDULLARY NEURONS

Fish	Sucrose-^{14}C space	Dry weight 10^{-12} kg	Volume 10^{-12} liter	Density	Na	K	Na	K	Na/K
					mmole/100 g dry		*mmole/liter per tissue volume*		
1		38.9	150	0.259	42		109		
		37.4	159	0.235	50		117		
2		40.1			57		128*		
		17.1			49		111*		
		45.6			44		100*		
		37.1			25		56*		
	4.72 {	42.4			55		123*		
		35.9			51		115*		
		49.4			32		73*		
		160			56		126*		
3	2.16	32.2	218	0.147	56	68	82	100	0.82
		53.9	231	0.233	46	76	108	177	0.61
		65.5	293	0.223	44	86	98	191	0.51
		81.9	366	0.224	49	77	109	172	0.63
		43.0	205	0.201	41	70	82	141	0.58
4	2.15	37.5	136	0.276	41	52	113	143	0.79
		38.7	165	0.234	59	48	138	113	1.22
		50.0	215	0.232	58	55	134	127	1.06
		35.0	132	0.265	43	51	114	136	0.84
		27.5	117	0.235	48	47	113	110	1.03
		21.3	83	0.256	37	43	94	109	0.86
5		46.9	224	0.209	56	64	116	134	0.87
		46.5	212	0.219	39	62	85	136	0.63
		55.9	281	0.199	47	79	94	158	0.59
		27.1	126	0.215	35	49	76	105	0.72
		48.3	204	0.236	28	44	65	103	0.63
		105	563	0.187	41	69	77	129	0.60
	3.01			0.225	45.51	61.17	102.1	134.4	0.76
				±0.007	±1.17	±3.30	±4.1	±6.6	±0.05

* Calculated assuming $D = 0.225$.

have studied the time course of sucrose accumulation between 15 and 90 min. The sucrose space within the cytoplasm is not altered within this period of time. Thus, there does not appear to be any evidence of active accumulation of the sucrose by such mechanisms as pinocytosis. The supramedullary neurons are close enough to the surface so that the sucrose could have diffused

within whatever space it occupies in the neurons within the first 15 min. Chromatography of TCA extracts of the tissue shows that the ^{14}C label is entirely within the sucrose peak, with no evidence of ^{14}C in the metabolic intermediates measured. There are no known enzymes capable of breaking down the sucrose within the brain of vertebrates. We have carried out autoradiography of freeze-dried sections which show a light scattering of silver grains over

TABLE III
NUCLEI OF SUPRAMEDULLARY NEURONS OF THE PUFFER FISH

Fish	Sucrose-^{14}C space	Dry weight 10^{-12} kg	Volume 10^{-12} liter	Density	Na	K	Na	K	Na/K
					mmole/100 g dry		mmole/liter per tissue volume		
1		35.9	166	0.216	51		110		
		33.7	150	0.224	38		85		
		13.4	86	0.156	26		40		
2	1.71	29.3			65		99*		
		45.0			44		67*		
		36.5			88		135*		
		22.4			75		114*		
		12.7			64		98*		
3	0.88	19.1	130	0.147	60	88	88	129	0.68
		40.2	278	0.144	47	83	68	119	0.57
		18.3	146	0.125	42	93	52	116	0.45
		48.2	360	0.134	60	127	80	151	0.53
		20.2	149	0.135	60	92	81	124	0.65
4	2.10	11.3	78	0.145	55	72	80	105	0.76
		21.3	150	0.142	60	80	85	114	0.75
5		51.7	344	0.155	67	81	104	126	0.82
		31.6	218	0.145	45	67	66	97	0.68
		23.9	185	0.129	53	65	69	84	0.82
		30.8	220	0.140	76	54	106	76	1.39
				0.153 ±0.008	56.63 ±3.38	82.00 ±5.78	85.6 ±5.3	112.8 ±6.4	0.74 ±0.07

* Calculated assuming $D = 0.153$.

the cytoplasm in a relatively uniform manner. It has not been possible on the autoradiographs to determine whether or not these grains are localized within any subcellular system. These results are reported elsewhere (Lehrer, 1969).

Na and K Content of Supramedullary Neurons

The detailed analyses of the Na and K content of the fragments of cytoplasm, nuclei, and perineural tissue dissected from the freeze-dried sections are

shown in Tables II–IV. In addition, the results are summarized for individual fish in Table V. Both sets of values have been included, since in some instances the variation within a fish was greater than the variations among fishes, and in some instances the reverse was true. However, it can be seen that the

TABLE IV

PERINEURONAL FRAGMENTS

	Fish	Sucrose-^{14}C space	Dry weight 10^{-12} kg	Volume 10^{-12} liter	Density	Na	K	Na	K	Na/K
						mmole/100 g dry		*mmole/liter per tissue volume*		
	1	10.3	133	683	0.195	36		70		
		7.5	191	1010	0.189	39		73		
		10.7	86	481	0.179	37		67		
			74	359	0.206	25		51		
			59	262	0.225	29		66		
	2		56.9			46		93*		
			46.2			37		75*		
			91.8			27		55*		
			101			37		76*		
			159			33		67*		
	3	9.7	123	640	0.192	59	62	114	120	0.95
			76	418	0.182	63	75	114	138	0.83
		9.5	111	615	0.181	55	74	99	134	0.74
			125	602	0.208	52	67	109	139	0.78
		7.7	135	870	0.155	46	46	72	72	1.00
	5		34	132	0.257	39	42	100	109	0.92
			57.8	276	0.209	40	52	83	109	0.76
			56.7	238	0.238	42	53	99	126	0.78
			56.7	233	0.244	38	54	93	132	0.70
			48.9	241	0.203	43	50	88	102	0.86
		9.23			0.204 ±0.007	41.15 ±2.25	57.5 ±3.62	83.2 ±4.2	118.1 ±4.2	0.83
Outer surface	3	21.1	154	718	0.214			72	128	

* Calculated assuming $D = 0.204$.

means and standard errors obtained in these two calculations do not materially differ from one another.

The Na concentration of the cytoplasmic fragments of supramedullary neurons was found to be 102.1 mmoles/liter tissue volume as compared to the value of 83.1 found for whole tissue. This was unexpected, since it had been anticipated that the Na content of the cytoplasm would be much less than

this,, perhaps of the order of 15 or 20 mmoles/liter tissue volume. The K concentration of 134.4 for the cytoplasm was similar to that of the whole tissue value of 128.8 as had been anticipated. Fragments of nucleus had somewhat less Na, 85.6 mmoles/liter tissue volume, and K, 112.8. The perineuronal fragments had a Na and K content very similar to that of the nuclei.

TABLE V
ANALYSES OF SUPRAMEDULLARY NEURONS. SUMMARY OF FISH

	Fish	No.	*D*	Sucrose space	Na	K	Na/K
					mmole/liter tissue volume		
Nucleus	1	3	0.199		78.3		
	2	5		1.71	102.6		
	3	5	0.137	0.88	73.8	127.8	0.58
	4	2	0.144	2.10	82.5	109.5	0.75
	5	4	0.142		86.3	95.7	0.93
			0.155 ±0.0146		84.7 ±4.9	111.0	0.75
Cytoplasm	1	2	0.247		113.0		
	2	8		4.72	104.0		
	3	5	0.205	2.16	95.8	156.2	0.63
	4	6	0.250	2.15	117.6	123.0	0.97
	5	6	0.211		85.5	127.5	0.67
			0.210 ±0.0117		103.1 ±5.8	135.6	0.76
Perineural tissue	1	5	0.199	9.5	65.4		
	2	5		9.25	73.2		
	3	5	0.184	8.9	101.6	120.6	0.86
	5	5	0.230		92.6	115.6	0.80
			0.204		83.2 ±8.4	118.1	0.83

Results of Metabolic Substrates

The lactic acid content of the sections from *S. maculatus* was 2.87 ± 0.09 mmoles/liter, and the phosphocreatine content was 5.25 ± 0.47 mmoles/liter. The concentration of phosphocreatine is the same or higher and the lactic acid concentration the same or lower than that reported for other vertebrate species, including goldfish well-adapted to their environment (McDougal et al., 1968). Hence, the puffer fish appear to have been well-oxygenated. The phosphocreatine falls rapidly, and lactic acid concentration rises rapidly in hypoxia.

DISCUSSION

Density

The finding that the nuclear fragments had a density of 0.15, whereas the cytoplasmic fragments had a density of 0.2 is perhaps surprising, since nuclei are often considered to be a dense portion of a cell. Indeed, in density centrifugation techniques, nuclei band at a higher density than do other cellular fractions. However, as Anderson (1966) has pointed out, during such centrifugations the nuclei go through many changes in size as they respond osmotically to their environment. Hence, their apparent density during this procedure will not reflect their density in vivo. Hyden and Larsson (1956) measured the nuclei, cytoplasm, and dendrite density in freshly frozen cells of Deiters's nucleus by X-radiograms. According to their data, the cytoplasmic density varied between 0.27 and 0.30 in one series and 0.25 in another; the dendrite, 0.28 and 0.20, and the nuclei, 0.29 and 0.20. It is, of course, probable that the density of nuclei will vary from cell to cell. Thus, DNA is one of the heaviest components of cells, and smaller nuclei with a higher DNA concentration may well have a higher density than very large nuclei with smaller DNA concentrations. In the supramedullary neuron, the nucleus is large, often measuring 100 μ in diameter. According to Nakajima et al. (1965), on electron microscopy the cytoplasm of the supramedullary neuron appears to be very dense compared to the nuclei.

Na and K Content of Supramedullary Neurons

The high Na concentration in cytoplasmic and nuclear fragments of supramedullary neurons was unexpected. Initially, when it became apparent that in vertebrate brain the high tissue Na could not be explained by the extracellular Na, one of us speculated that this high Na might be inside astrocytic glia (Katzman, 1961). This speculation was based in part on the high Na content of abnormal glia found in glial tumors, glial scars, or edematous brain. In the intervening years, a body of evidence has accumulated pointing to a high K content of glial cells in certain vertebrates as well as in the leech (Kuffler, 1967). In view of this work and our present findings, it would appear that excess intracellular Na need not be attributed to high glial Na.

It is worthwhile to compare our present results with other measurements of intracellular neuronal Na in vertebrates. Fenn et al. in 1934 calculated that the intracellular Na of the frog nerves amounted to 37.4 mmoles/kg of water and the intracellular K, 172.9 mmoles/kg of water. This calculation was based on the assumption that there was an extracellular space corresponding to the Na and Cl present and an intracellular space that was primarily K. Krnjevic (1955) estimated the intracellular Na in cat nerves to be 41 mmoles/kg of water; K, 182, and Cl, 0 to 17.5. This estimate was based upon measurements

of Na and K content of intact and desheathed nerves with the extracellular space of 56.6 % estimated from photomicrographs. If the inulin space, which was 11 % in these nerves, had been used to calculate extracellular space, the intracellular Na would have been much larger. Müller (1958) was the first to directly measure the Na and K content of single vertebrate axons. He measured the Na and K content of single myelinated fibers of frogs using an integrating microflame photometer similar to the one reported here. The Na concentration varied between 50 and 80 mM and the K between 50 and 145 mM. It is worthwhile noting the large variation between different fibers which to a large extent is also true in the analyses reported in the present work. Keesey (1968) also using an integrating microflame photometer measured the Na and K in nanogram samples of neural tissue dissected from the rabbit thoracic ganglion, rapidly frozen, lyophilized, and weighed on a quartz fiber balance. The Na content of one ganglion was 27.8 mmoles/100 g dry weight and in a second ganglion 21.9 mmoles/100 g dry weight. This should be compared with our results of 56.6 mmoles/100 g dry weight in nuclei and 45.5 mmoles/100 g dry weight in cytoplasm. Thus, the intracellular Na concentration measured in both these experiments was much greater than had been anticipated.

In their classic study of the electrophysiology of these neurons, Bennett et al. (1959 *a*) showed that these neurons had membrane characteristics similar to those of other neurons and generated potentials with overshoots suggesting that an increase in Na conductance was associated with the spike potential. It has been assumed that the intracellular concentration of free Na is low in neurons, e.g., 15 mM in the cat motoneuron according to Eccle's estimate (1957). Let us consider possible explanations of the unexpectedly high Na concentrations obtained in our analyses of the supramedullary neuron.

1. The presumed intracellular Na actually represents Na from invaginating capillaries and glial cells. To test this theory, analyses were carried out on severely trimmed fragments of cytoplasm. The data of Nakajima et al. (1965) show that the invaginating glial processes and capillaries are heavily concentrated in the outer third of the circumference of the cell. There appeared to be no significant difference between the Na content of either whole cells, severely trimmed cells, or cytoplasm removed from the perinuclear region of the cell. Moreover, the microprobe data did not show any significant Na gradient between the perinuclear cytoplasm and the cytoplasm just adjacent to the plasma membrane where the highest concentrations of intracellular capillaries and glial cells are located.[4]

2. The high Na is located within endoplasmic reticulum which is open to the exterior of the cell and represents an invaginating tubular system contain-

[4] Lehrer, G. M., and A. Langer. Data to be published.

ing extracellular fluid. This possibility is indeed a plausible one. If it were applicable to mammalian tissue, it would clarify the discrepancies between extracellular spaces measured with sucrose or inulin tags and extracellular spaces observed on electron micrographs.[3] In order to check this hypothesis, the sucrose space was measured and was found to be 3 %. If this value is ascribed to an extracellular space, and it is assumed that this extracellular space contains Na at 150 mM, the resulting correction of the intracellular cytoplasmic Na value will be less than 2 mM, not significantly altering our finding.

3. Another possibility is that intracellular Na is sequestered within organelles. Under suitable conditions, mitochondria may accumulate Na. In the preparations that have been studied under normal aerobic conditions, such Na accumulation by mitochondria does not appear to be large enough to account for the discrepancy between Na as measured by flame photometry and Na as estimated from electrophysiological studies. There is virtually no information about Na binding to other subcellular structures.

4. A substantial portion of intracellular Na is bound. The limit of free intracellular Na permitted by the Hodgkin-Huxley-Keynes hypothesis can be determined from the overshoot of the action potential recorded intracellularly. Bennett et al. (1959 *a*, *b*) reported that the resting potential in the supramedullary neuron was 66 ± 10 mv with a spike height of 92 ± 19 mv. This gives an average spike overshoot of 26 mv. Looking at their individual numbers, the largest overshoot was 42 mv. The calculation of the equilibrium potential of Na responsible for the spike potential is based upon Na activity rather than Na concentration. In order to account for this average overshoot in spike height the ratio of extracellular to free intracellular Na should be 3.5 to 1, considering the temperature at which these measurements were made. The difference between the measured Na content and the concentration calculated from the overshoot is probably due to the fact that Na ions are bound to organic molecules within the cell. Recently, Cope (1967) has presented nuclear magnetic resonance evidence for complexing of Na in muscle, kidney, and brain. Estimates of the fraction of bound Na in squid axoplasm vary between 25 and 50%. However, the degree of such binding within neuronal perikaryon has not been measured.

There is considerable evidence that Na may be bound to organic molecules. A number of proteins and organic molecules broaden and decrease the height of the Na peak measured by nuclear magnetic resonance. Perhaps a more direct demonstration of the ability of organic molecules to lower Na activity was the demonstration by Christensen and Hastings (1940) that a Na "cephalin" suspension containing an acidic lipid, probably phosphatidylserine, had a low Na activity coefficient as measured with a Na amalgam electrode. Essentially, the Na was bound to its counterion, phosphatidylserine, and the Na

activity in the suspending medium was negligible. Within the cell, however, the situation is more complex, because the Na is readily displaced from acidic lipids by Ca and Mg. However, it is interesting that the formation constant of Na with such molecules as phosphatidylserine and phosphatidic acid is about twice that for K (Abramson et al., 1966).

Recent measurements of intracellular Na activity have utilized Na-sensitive glass microelectrodes. Lev (1964) found Na activity coefficients in frog muscle fibers to be only 0.20 compared to a K activity coefficient of 0.77. McLaughlin and Hinke (1966) in the giant barnacle muscle fiber found Na activity to be only 16 % of the total Na concentration. In contrast, the Na activity coefficient of the extruded axoplasm of the squid giant axon was higher, 0.46 (Hinke, 1961). This apparent inconsistency might be due in part to the fact that RNA, a major polyanion in the perikaryon, is low in axons. In view of these data, it would be of considerable interest to measure the Na activity within the supramedullary neuron by use of the glass electrode. From the data on hand, we predict that over one-half of the intracellular Na should be bound in the cytoplasm of the puffer fish.

The authors gratefully acknowledge the assistance of Miss Anna Yam and Mr. Emil Stephenson.
This work was supported by grants NB 01450, NB 03356, and NB 05368, United States Public Health Service, and grant 293, National Multiple Sclerosis Society.

Received for publication 7 February 1969.

REFERENCES

Abramson, M. B., R. Katzman, H. Gregor, and R. Curci. 1966. The reactions of cations with aqueous dispersions of phosphatidic acid. Determination of stability constants. *Biochemistry.* **5**:2207.

Anderson, N. G. 1966. An introduction to particle separations in zonal centrifuges. *In* The Development of Zonal Centrifuges and Ancillary Systems for Tissue Fractionation and Analysis. N. G. Anderson, Editor. National Cancer Institute Monograph. U. S. Government Printing Office, Washington, D. C. pp. 9–40.

Bennett, M. V. L., S. M. Crain, and H. Grundfest. 1959 *a*. Electrophysiology of supramedullary neurons in *Spheroides maculatus*. I. Orthodromic and antidromic responses. *J. Gen. Physiol.* **43**:159.

Bennett, M. V. L., S. M. Crain, and H. Grundfest. 1959 *b*. Electrophysiology of supramedullary neurons in *Spheroides maculatus*. II. Properties of the electrically excitable membrane. *J. Gen. Physiol.* **43**:219.

Bennett, M. V. L., S. M. Crain, and H. Grundfest. 1959 *c*. Electrophysiology of supramedullary neurons in *Spheroides maculatus*. III. Organization of the supramedullary neurons. *J. Gen. Physiol.* **43**:221.

Bennett, M. V. L., Y. Nakajima, and G. D. Pappas. 1967. Physiology and ultrastructure of electrotonic junctions. I. Supramedullary neurons. *J. Neurophysiol.* **30**:161.

Bott, P. A. 1960. The determination of sodium and potassium in biological fluids with the dual channel ultramicro flame photometer. *Anal. Biochem.* **1**:17.

Carlsson, B., E. Giacobini, and S. Hovmark. 1967. An instrument for simultaneous determination of sodium and potassium in microsamples of biological material. *Acta Physiol. Scand.* **71**:379.

Christensen, H. N., and A. B. Hastings. 1940. Phosphatides and inorganic salts. *J. Biol. Chem.* **136**:387.

COPE, F. W. 1967. NMR evidence for complexing of Na^+ in muscle, kidney, and brain, and by actomyosin. The relation of cellular complexing of Na^+ to water structure and to transport kinetics. *J. Gen. Physiol.* **50**:1353.

ECCLES, J. C. 1957. The Physiology of Nerve Cells. The Johns Hopkins University Press, Baltimore.

FENN, W. O., D. M. COBB, A. H. HEGNAUER, and B. S. MARSH. 1934. Electrolytes in nerve. *Amer. J. Physiol.* **110**:74.

HINKE, J. A. M. 1961. The measurement of sodium and potassium activities in the squid axon by means of cation-selective glass micro-electrodes. *J. Physiol.* (*London*). **156**:314.

HYDEN, H., and S. LARSSON. 1956. The application of a scanning and computing cell analyzer to neurocytological problems. *J. Neurochem.* **1**:134.

KATZMAN, R. 1961. Electrolyte distribution in mammalian central nervous system. Are glia high sodium cells? *Neurology.* **11**:27.

KEESEY, J. C. 1968. Flame photometric analysis of sodium and potassium in nanogram samples of mammalian nervous tissue. *J. Neurochem.* **15**:547.

KRNJEVIC, K. 1955. The distribution of Na and K in cat nerves. *J. Physiol.* (*London*). **128**:473.

KUFFLER, S. W. 1967. Neuroglial cells: Physiological properties and a potassium mediated effect of neuronal activity on the glial membrane potential. *Proc. Roy. Soc. Ser. B. Biol. Sc.* **168**:1.

KUFFLER, S. W., AND J. G. NICHOLLS. 1966. The physiology of neuroglial cells. *Ergeb. Physiol.* **57**:1.

LEHRER, G. M. 1969. The central nervous system sucrose space—A comparison of quantitative and radioautographic data. *In* International Conference on High Resolution Autoradiography of Diffusible Substances. L. J. Roth, editor. Academic Press, Inc., New York. P. 191.

LEHRER, G. M., C. WEISS, D. J. SILIDES, C. LICHTMAN, M. M. FURMAN, and R. F. MATHEWSON. 1968. The quantitative histochemistry of supramedullary neurons of puffer fishes. *J. Cell Biol.* **37**:575.

LEV, A. A. 1964. Determination of activity and activity coefficients of potassium and sodium ions in frog muscle fibres. *Nature* (*London*). **201**:1132.

LOWRY, O. H. 1953. The quantitative histochemistry of the brain. Histological sampling. *J. Histochem. Cytochem.* **6**:420.

MAKER, H. S., G. M. LEHRER, C. WEISS, D. J. SILIDES, and L. C. SCHEINBERG. 1966. The quantitative histochemistry of a chemically induced ependymoblastoma. II. The effect of ischemia on substrates of carbohydrate metabolism. *J. Neurochem.* **13**:1207.

MCDOUGAL, D. B., JR., J. HOLOWACH, M. C. HOWE, E. M. JONES, and C. A. THOMAS. 1968. The effects of anoxia upon energy sources and selected metabolic intermediates in the brains of fish, frog and turtle. *J. Neurochem.* **15**:377.

MCLAUGHLIN, S. G. A., and J. A. M. HINKE. 1966. Sodium and water binding in single striated muscle fibers of the giant barnacle. *Can. J. Physiol. Pharmacol.* **44**:837.

MÜLLER, P. 1958. Experiments on current flow and ionic movements in single myelinated nerve fibers. *Exp. Cell Res.* **152**:118.

NAKAJIMA, Y., D. G. PAPPAS, and M. V. L. BENNETT. 1965. The fine structure of the supramedullary neurons of the puffer with special reference to endocellular and pericellular capillaries. *Amer. J. Anat.* **116**:471.

OBERG, P. A., H. R. ULFENDAHL, and B. G. WALLIN. 1967. An integrating flame photometer for simultaneous microanalysis of sodium and potassium in biological fluids. *Anal. Biochem.* **18**:543.

RAMSEY, J. A., R. H. J. BROWN, and W. H. W. FALLOON. 1953. Simultaneous determination of sodium and potassium in small volumes of fluid by flame photometry. *J. Exp. Biol.* **30**:1.

VAN HARREVELD, A. 1966. Water and electrolyte distribution in normal and abnormal central nervous tissue. *In* Head Injury. W. F. Caveness and A. E. Walker, editors. J. B. Lippincott Company, London. P. 397.