Tetrodotoxin-Sensitive Uptake of Ions and Water by Slices of Rat Brain *in vitro*

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1. Under certain conditions the processes that bring about increased Na^+ influx and water uptake into rat brain slices have a tetrodotoxin-sensitive component. 2. Incubation of slices in the absence of glucose, or in the presence of ouabain (0.1 mM), protoveratrine (10 μM) or electrical stimuli, leads to increased uptakes of water and Na^+ that are partially suppressed by tetrodotoxin (3 μM). 3. The increased water uptake and Na^+ influx due to the presence of 30 μM -2,4-dinitrophenol or of 100 mM-potassium chloride are unaffected by tetrodotoxin. 4. The additional presence of 27 mM-potassium chloride diminishes, or abolishes, the inhibitory effects of tetrodotoxin on the increased uptakes of water and Na^+ found on incubation in the absence of glucose or in the presence of protoveratrine. 5. Ouabain increases Na^+ influx into caudate nuclei from rat brain and this process is suppressed by tetrodotoxin. Ouabain does not increase water uptake in the caudate nuclei. 6. Diminution of Na^+ influx in slices due to tetrodotoxin is not accompanied by an equal efflux of K^+ . 7. It is concluded that glial-cell membranes are permeable to K^+ and Cl^- and, as a consequence, when K^+ is released from neurons in association with action potentials it, together with water, moves into the glial cells. The major consequence of K^+ (and Cl^-) permeability of the glial cell is therefore a buffering of the extracellular K^+ concentration. 8. L-Glutamate presents an anomalous feature that may indicate that its effects are not restricted to neurons. 9. Calculations are made of the increase of K^+ flux into the glia in the presence of ouabain or of protoveratrine or in the absence of glucose or on application of electrical impulses.

Results of experiments described in the preceding paper (Okamoto & Quastel, 1970) have indicated that substances or conditions leading to changed ion fluxes in incubated slices of brain cortex from the rat may be divided into two groups. One group affects directly the permeability of the cell membrane to Na^+ and another group affects the ionic fluxes indirectly by modification of the operation of the sodium pump either by effects on the activity of Na^+ + K^+ -stimulated adenosine triphosphatase or on the brain-cell ATP concentration.

We have carried out experiments to observe whether it is possible to discriminate between these two groups by the use of tetrodotoxin, which is well known to abolish the generation of action potentials at the neuronal membrane and hence the associated influx of Na^+ (Kao, 1966; Narahashi, Moore & Scott, 1964; Nakamura, Nakajima & Grundfest, 1965). Our experiments indicate that there exist tetrodotoxin-sensitive components of

the increased Na^+ and water uptake into brain cells that accompany a variety of incubation conditions. The results of these experiments and their possible implications in our understanding of cerebral ion fluxes are described below.

MATERIALS

Animals. Male adult rats, usually more than 2 months old, of the Wistar strain weighing 150-200 g were used. For certain experiments, described in the text, animals only 20 days old were used. All animals were obtained from the Vivarium, or the Animal Unit, University of British Columbia, Vancouver, B.C., Canada.

Radioactive material. $^{22}\text{NaCl}$ of specific radioactivity 2.3 mCi/ μmol was obtained in aqueous solution (0.1 mCi/ml) from The Radiochemical Centre, Amersham, Bucks., U.K.

METHODS

The preparation of rat brain slices, the incubation procedure, the preparation of different incubation media, the method of electrical stimulation, the determination

of water uptake and of the tissue content of radioactive Na^+ are all fully described in the preceding paper (Okamoto & Quastel, 1970).

In a number of experiments the tissue contents of unlabelled Na^+ and K^+ were determined with an atomic absorption spectrophotometer. These assays were carried out as follows. The incubated slices were homogenized in 3 ml of 5% (w/v) trichloroacetic acid and the supernatants obtained after centrifugation were diluted with water to obtain final cation concentration of about 0.05–0.10 $\mu\text{equiv./ml}$ for both Na^+ and K^+ . The atomic absorptions were measured at 294.3 and 383.3 nm for Na^+ and K^+ respectively with a Perkin–Elmer model 303 atomic-absorption spectrophotometer.

Number of experiments. All experiments, results of which are given below, were carried out at least three times. The means of these results $\pm$ s.d. are recorded.

RESULTS

Relations between water uptake and concentrations of Na^+ and K^+ in incubated adult rat brain-cortex slices

Effects of ouabain, protoveratrine and absence of glucose. When brain-cortex slices from adult rats are incubated aerobically for 1 h in a glucose-containing medium in the presence of ouabain (0.1 mM), there is an increased uptake of water in the slices, an increased concentration of Na^+ and a diminished concentration of K^+ . The results given in Table 1 show that the net change in total Na^+ and K^+ in the tissue, due to the incubation with ouabain, is a gain of 30.3 $\mu\text{equiv./g}$ initial wet wt. The gain in weight of water is $392 - 188 = 204 \mu\text{l/g}$

initial wet wt. Assuming that the fluid entering the tissue is iso-osmotic with the incubation medium, the cation ($\text{Na}^+ + \text{K}^+$) content of the water taken up $= 204 \times 0.153 = 31.2 \mu\text{equiv./g}$ initial wet wt. (as the incubation medium containing 148 mequiv. of Na^+/l and 5 mequiv. of K^+/l). This value is identical, within experimental error, with the observed change in cation ($\text{Na}^+ + \text{K}^+$) content of the tissue and supports the conclusion that water is taken up to maintain iso-osmoticity (Leaf, 1956).

Incubation of brain tissue with protoveratrine (5 μM) leads to an increased concentration of Na^+ and a decreased concentration of K^+ , the net change in total Na^+ and K^+ being so small (0.5 $\mu\text{equiv./g}$) as not to be significant. The change in water uptake is also so small as not to be significant (Table 1).

Incubation of the tissue in the medium in the absence of glucose leads to a marked uptake of water with an accompanying increased concentration of Na^+ and a decreased concentration of K^+ in the tissue. The change in total Na^+ and K^+ in the tissue amounts to an increase of 35.9 $\mu\text{equiv./g}$. The uptake of water $= 415 - 188 = 227 \mu\text{l/g}$, which, for iso-osmoticity with the medium, will have a total cation ($\text{Na}^+ + \text{K}^+$) content of $227 \times 0.153 = 34.7 \mu\text{equiv./g}$. There is therefore identity, within experimental error, between observed and calculated values of the total cationic ($\text{Na}^+ + \text{K}^+$) changes due to incubation of the tissue in the absence of glucose.

The total concentration of Na^+ and K^+ in the

Table 1. Concentrations of Na^+ and K^+ and estimates of water uptake in adult rat brain-cortex slices incubated under a variety of conditions

Incubation was carried out in O_2 for 1 h at 37°C in Krebs–Ringer phosphate medium, as described in the text, with or without additions as specified below. Na^+ and K^+ were assayed at the end of the experiment with a Perkin–Elmer atomic-absorption spectrophotometer. A + sign in the sixth column indicates an increase in the total $\text{Na}^+ + \text{K}^+$ in the tissue after incubation above that in the control. The next column shows the increased amount of Na^+ entering the tissue, after incubation, calculated on the basis of the increased fluid uptake being iso-osmotic with the incubation medium.

Substances added to incubation medium	O_2 uptake ($\mu\text{mol}/100\text{mg}$ initial wet wt.)	Water uptake ($\mu\text{l}/100\text{mg}$ initial wet wt.)	Na^+ content ($\mu\text{equiv./g}$ initial wet wt.)	K^+ content ($\mu\text{equiv./g}$ initial wet wt.)	Change of total $\text{Na}^+ + \text{K}^+$ from that in the control ($\mu\text{equiv./g}$ initial wet wt.)	Na^+ equivalent to increase of water uptake	Concn. of $\text{Na}^+ + \text{K}^+$ in the tissue (mequiv./l)
10 mM-Glucose (control)	9.1 ± 0.3	18.8 ± 2.4	112.1 ± 1.0	54.8 ± 2.0	—	—	$\frac{166.9}{0.988} = 169$
10 mM-Glucose + 0.1 mM-ouabain	9.1 ± 0.5	39.2 ± 0.3	168.3 ± 0.5	28.9 ± 0.1	+30.3	+31.2 (20.4×1.53)	$\frac{197.2}{1.192} = 166$
10 mM-Glucose + 5 μM -protoveratrine	12.3 ± 0.4	18.5 ± 1.3	129.5 ± 3.5	36.9 ± 0.3	-0.5	-0.5 (0.3×1.53)	$\frac{166.4}{0.985} = 169$
No glucose added	6.0 ± 0.5	41.5 ± 1.0	181.5 ± 1.0	21.3 ± 0.9	+35.9	+34.7 (22.7×1.53)	$\frac{202.8}{1.215} = 167$

Table 2. *Rate of uptake of $^{22}\text{Na}^+$ by incubated rat brain-cortex slices*

Incubation was carried out in O_2 at 37°C in Krebs-Ringer phosphate medium containing 10mm-glucose. $^{22}\text{NaCl}$ ($0.33\mu\text{Ci/ml}$ of medium) was tipped from the side tube of the manometric vessel into the medium 5min after incubation had commenced. $^{22}\text{Na}^+$ in the tissue slices and the water uptake of the slices were assayed at the time-intervals noted below.

Incubation time (min)	Water uptake ($\mu\text{l}/100\text{mg}$ initial wet wt.)	$^{22}\text{Na}^+$ uptake ($\mu\text{equiv.}/\text{g}$ initial wet wt.)
1	7.3	52.6
2	7.7	61.3
4	7.6	85.0
6	9.7	95.2
10	10.7	96.2
30	15.9	104.0
60	20.3	110.6

tissue, calculated from the results given in Table 1 and taking the initial water content of the brain slices to be 80%, is approx. 168mm for all conditions (Table 1). It is therefore 15mequiv./l higher than the external concentration (153mequiv./l).

Uptake of $^{22}\text{Na}^+$ by incubated brain slices. The addition of $^{22}\text{NaCl}$ ($0.33\mu\text{Ci/ml}$ of medium), amounting to 0.14nmol/ml , to the medium in which slices from adult rats are being incubated aerobically at 37°C leads to a rapid exchange between the labelled and unlabelled Na^+ in the slices. The rates of uptake of $^{22}\text{Na}^+$ and of water are shown in Table 2. Almost 80% of the maximum uptake of $^{22}\text{Na}^+$ takes place within 4min after the commencement of incubation. Within the incubation period of 1h all the $^{22}\text{Na}^+$ exchanges with the unlabelled Na^+ in the slice, as is seen on comparing the values of Na^+ ($112.1\mu\text{equiv.}/\text{g}$) (Table 1) and $^{22}\text{Na}^+$ ($110.6\mu\text{equiv.}/\text{g}$) (Table 2).

An immediate uptake of water, amounting to about 7%, is followed by a slower uptake of water, giving a value of approx. 20% after 1h incubation. These results are similar to those found by Varon & McIlwain (1961) and by Keeseey & Wallgren (1965) with incubated slices from guinea-pig brain.

Effects of tetrodotoxin on the electrically stimulated uptakes of water and $^{22}\text{Na}^+$ in incubated brain slices. The application of electrical stimuli to brain slices from adult rats incubated aerobically in a glucose medium for 1h results in increases of water uptake and of $^{22}\text{Na}^+$ concentration in the tissue. The presence of tetrodotoxin ($3\mu\text{M}$), which has no effect, within the experimental error, on the water uptake and the $^{22}\text{Na}^+$ concentration in the absence of electrical stimuli, brings about almost a complete block of both the increased water uptake and

$^{22}\text{Na}^+$ concentration that occurs on electrical stimulation. The results given in Table 3 show that the presence of tetrodotoxin brings about a diminution of the $^{22}\text{Na}^+$ uptake, in electrically stimulated slices, of $15.3\mu\text{equiv.}/\text{g}$. It also brings about a diminution of water uptake in these slices of $81\mu\text{l}/\text{g}$, which will contain, for iso-osmoticity with the medium, $81 \times 0.153 = 12.4\mu\text{equiv.}/\text{g}$. It therefore appears that the decrease in cation ($\text{Na}^+ + \text{K}^+$) influx, accompanying the diminution of water uptake brought about by tetrodotoxin acting on electrically stimulated slices, corresponds to the decrease in $^{22}\text{Na}^+$ entry found under these conditions.

Also, tetrodotoxin suppresses the increase in the rate of oxygen consumption brought about by electrical stimulation (Table 3), a fact shown earlier by Chan & Quastel (1967a,b).

Effects of tetrodotoxin on the uptakes of water and on the concentrations of $^{22}\text{Na}^+$ in brain-cortex slices from adult rats incubated under a variety of conditions

Ouabain. Ouabain (0.1mm) brings about a large influx of Na^+ into incubated slices accompanied by an increased water uptake, a fact already established (e.g. Yoshida, Nukada & Fujisawa, 1961; Bourke & Tower, 1966a,b). Results given in Table 4 show that both the uptake of water and the influx of Na^+ , brought about by ouabain, have a tetrodotoxin-sensitive component. Tetrodotoxin ($3\mu\text{M}$) lowers the increased uptake of $^{22}\text{Na}^+$ from 59.4 to $33.1\mu\text{equiv.}/\text{g}$ and the increased uptake of water from 232 to $73\mu\text{l}/\text{g}$ under the experimental conditions quoted. The diminution of water uptake, amounting to $159\mu\text{l}/\text{g}$, corresponds to a diminished cation influx ($\text{Na}^+ + \text{K}^+$) of $159 \times 0.153 = 24.3\mu\text{equiv.}/\text{g}$ for maintenance of iso-osmoticity. This value approximates to the observed diminution, $26.3\mu\text{equiv.}/\text{g}$, of $^{22}\text{Na}^+$ influx.

Protoveratrine. Veratrine alkaloids bring about marked changes in ion permeability and bioelectric phenomena (Shanes, 1958a,b), and protoveratrine affects the kinetics of glucose metabolism in incubated brain slices (Wollenberger, 1955a,b; Kini & Quastel, 1960), presumably by changes in ion permeability. Consistent with this fact it is seen from the results quoted in Table 4 that protoveratrine ($10\mu\text{M}$) increases the influx of $^{22}\text{Na}^+$ and, to a relatively small extent, the uptake of water by incubated rat brain-cortex slices. The increased uptakes of $^{22}\text{Na}^+$ and of water are abolished by the presence of tetrodotoxin ($3\mu\text{M}$). The decrease in $^{22}\text{Na}^+$ concentration due to tetrodotoxin, in the presence of protoveratrine, amounts to $29.8\mu\text{equiv.}/\text{g}$, whereas the diminution of water uptake amounts to $125\mu\text{l}/\text{g}$, equivalent, for iso-osmoticity,

Table 3. *Effects of tetrodotoxin on the uptakes of water and on the concentrations of $^{22}\text{Na}^+$ in incubated rat brain-cortex slices with or without electrical stimulation*

Incubation was carried out in O_2 for 1 h at 37°C in Krebs-Ringer phosphate medium containing 10 mM-glucose. $^{22}\text{Na}^+$ ($0.333 \mu\text{Ci/ml}$ of medium) was tipped into the medium from the side tube of the manometric vessel 5 min after incubation had commenced. Tetrodotoxin ($3 \mu\text{M}$) was added to the medium at the commencement of the experiment. Electrical stimuli were applied as described in the text. Values given in the sixth column are those in the fifth column multiplied by 1.53. For details see the text.

	O_2 uptake ($\mu\text{mol}/100\text{mg}$ initial wet wt.)	Water uptake ($\mu\text{l}/100\text{mg}$ initial wet wt.)	Change of water uptake from that in the control ($\mu\text{l}/100\text{mg}$ wet wt.)	Net effect of tetrodotoxin		$^{22}\text{Na}^+$ content ($\mu\text{equiv.}/\text{g}$ initial wet wt.)	Increase of $^{22}\text{Na}^+$ over that in the control ($\mu\text{equiv.}/\text{g}$ initial wet wt.)		Net effect of tetrodotoxin on increased cation ($\text{Na}^+ +$ K^+) uptake ($\mu\text{equiv.}/\text{g}$ wet wt.)	Net effect of tetrodotoxin on increased $^{22}\text{Na}^+$ uptake ($\mu\text{equiv.}/\text{g}$ wet wt.)
				on the increased water uptake ($\mu\text{l}/100\text{mg}$ wet wt.)	on the equivalent cation ($\text{Na}^+ +$ K^+) uptake ($\mu\text{equiv.}/\text{g}$ wet wt.)					
Incubation condition										
Control	9.0 ± 0.6	17.7 ± 2.0	—	—	—	111.4 ± 3.6	—	—	—	—
Tetrodotoxin added	8.6 ± 0.3	16.8 ± 1.8	-0.9	-0.9	-1.3	111.5 ± 0.2	0.1	0.1	—	0.1
Electrical stimuli applied	12.6 ± 0.3	24.6 ± 0.3	+6.9	—	—	130.8 ± 3.5	19.4	19.4	—	—
Electrical stimuli applied, added tetrodotoxin	9.0 ± 0.3	16.5 ± 3.0	-1.2	-8.1	-12.4	115.5 ± 4.5	4.1	4.1	-15.3	-15.3

to a decreased cationic influx ($\text{Na}^+ + \text{K}^+$) of $125 \times 0.153 = 19.1 \mu\text{equiv.}/\text{g}$.

It is evident also from results given in Table 4 that tetrodotoxin abolishes the increased rate of respiration of slices brought about by the presence of protoveratrine.

Effects of absence of glucose. As is already known, a diminished energy supply to the brain leads to uptake of Na^+ and of water in brain slices. Absence of glucose from the incubation medium brings about marked increases in the uptake of water and of $^{22}\text{Na}^+$ into slices incubated aerobically for 1 h at 37°C . Both the influx of $^{22}\text{Na}^+$ and the uptake of water have a tetrodotoxin-sensitive component. Results given in Table 4 show that an influx of $74.8 \mu\text{equiv.}$ of $^{22}\text{Na}^+/\text{g}$ due to absence of glucose is decreased to $60.6 \mu\text{equiv.}/\text{g}$ by the presence of tetrodotoxin, amounting to a fall of $14.2 \mu\text{equiv.}/\text{g}$. The increase of water uptake is decreased from 240 to $164 \mu\text{l}/\text{g}$ by tetrodotoxin, the decrease amounting to $76 \mu\text{l}/\text{g}$, equivalent to a fall in cation ($\text{Na}^+ + \text{K}^+$) uptake of $76 \times 0.153 = 11.6 \mu\text{equiv.}/\text{g}$. Results given in Table 5 show that tetrodotoxin has no significant effect on the tissue content of K^+ when the slices are incubated in the absence of glucose.

L-Glutamate. The well-known increased influx of Na^+ and of water due to the presence of L-glutamate in incubated nervous tissue (Ames, 1956; Curtis, Phillips & Watkins, 1960; Bourke & Tower, 1966a,b; Ames, Tsukada & Nesbitt, 1967) is seen in the results quoted in Table 4. Addition of tetrodotoxin ($3 \mu\text{M}$) brings about some diminution of the influx of $^{22}\text{Na}^+$, the increased uptake of $21.8 \mu\text{equiv.}$ of $^{22}\text{Na}^+/\text{g}$, due to the presence of L-glutamate, being decreased to $13.6 \mu\text{equiv.}/\text{g}$. This result is in accord with the observations of Pull, McIlwain & Ramsey (1970) with cortex slices from guinea-pig brain. However, tetrodotoxin, within the limits of experimental error, brings about little or no change in the water uptake under the given experimental conditions.

2,4-Dinitrophenol. The increased water uptake, $^{22}\text{Na}^+$ influx and rate of respiration found in slices in the presence of 2,4-dinitrophenol ($30 \mu\text{M}$) is unaffected under the given experimental conditions by the addition of tetrodotoxin ($3 \mu\text{M}$) (Table 6).

Effects of high concentration of K^+ . The increased uptakes of water and of $^{22}\text{Na}^+$ found when slices are incubated in the presence of added 100 mM-potassium chloride are unaffected by the presence of tetrodotoxin ($3 \mu\text{M}$) (Table 6). When 27 mM-potassium chloride is added to the normal incubation medium there is no increase in water uptake or $^{22}\text{Na}^+$ influx, and tetrodotoxin has no effect on these values within experimental error (Table 6). Presumably cortex slices from rat brain are not as sensitive to increased concentrations of K^+ as are

Table 4. *Effects of tetrodotoxin on the uptakes of water and on the concentration of $^{22}\text{Na}^+$ in rat brain-cortex slices incubated under a variety of conditions*

Incubation was carried out in O_2 for 1 h at 37°C in Krebs-Ringer phosphate medium as described in the text. Glucose (10 mM) was present (except where otherwise stated). $^{22}\text{Na}^+$ ($0.333\ \mu\text{Ci}/\text{ml}$ of medium) was tipped into the medium from the side tube of the manometric vessel 5 min after incubation had commenced. Tetrodotoxin ($3\ \mu\text{M}$) was added to the medium at the commencement of the experiment. The values given in the sixth column are calculated by multiplying the values in the fifth column by 1.53. For details see the text.

Substances added to incubation medium	O_2 uptake ($\mu\text{mol}/100\text{mg}$ initial wet wt.)	Water uptake ($\mu\text{l}/100\text{mg}$ initial wet wt.)	Change of water uptake from that in the control ($\mu\text{l}/100\text{mg}$ wet wt.)	Net effect of tetrodotoxin		$^{22}\text{Na}^+$ content ($\mu\text{equiv.}/\text{g}$ initial wet wt.)	Increase of $^{22}\text{Na}^+$ over that in the control ($\mu\text{equiv.}/\text{g}$ initial wet wt.)	Net effect of tetrodotoxin on increased $^{22}\text{Na}^+$ uptake ($\mu\text{equiv.}/\text{g}$)
				on the increased water uptake ($\mu\text{l}/100\text{mg}$ wet wt.)	on the equivalent cation ($\text{Na}^+ + \text{K}^+$) uptake ($\mu\text{equiv.}/\text{g}$ wet wt.)			
10 mM-Glucose (control)	10.2 ± 0.2	17.7 ± 1.2	—	—	—	107.6 ± 1.0	—	—
10 mM-Glucose + 0.1 mM-ouabain	8.6 ± 0.4	40.9 ± 0.9	+23.2	—	—	167.0 ± 0.4	59.4	—
10 mM-Glucose + 0.1 mM-ouabain + tetrodotoxin	8.3 ± 0.4	25.0 ± 1.9	+7.3	-15.9	-24.3	140.7 ± 3.3	33.1	-26.3
10 mM-Glucose + $10\ \mu\text{M}$ -protopoveratine	13.4 ± 0.3	25.4 ± 1.4	+7.7	—	—	134.6 ± 1.8	27.0	—
10 mM-Glucose + $10\ \mu\text{M}$ -protopoveratine + tetrodotoxin	10.1 ± 0.3	12.9 ± 0.2	-4.8	-12.5	-19.1	104.8 ± 3.4	-2.8	-29.8
Glucose absent	6.3 ± 0.2	43.7 ± 2.1	+26.0	—	—	182.4 ± 2.6	74.8	—
Tetrodotoxin; glucose absent	6.1 ± 0.3	33.9 ± 2.6	+16.2	-9.8	-14.9	168.2 ± 2.0	60.6	-14.2
10 mM-Glucose + 5 mM-L-glutamate	10.3 ± 0.3	38.5 ± 1.8	+20.8	—	—	129.4 ± 2.5	21.8	—
10 mM-Glucose + 5 mM-L-glutamate + tetrodotoxin	10.3 ± 0.3	37.9 ± 3.0	+20.2	-0.6	-0.9	121.2 ± 1.9	13.6	-8.2

Table 5. *Effects of tetrodotoxin (3 μ M) on Na⁺ and K⁺ contents in rat brain-cortex slices incubated in the absence of glucose*

Incubation was carried out in O₂ for 1 h at 37°C in Krebs-Ringer phosphate medium. No glucose was added. Na⁺ and K⁺ were determined in the brain-cortex slices at the termination of the incubation period with an atomic-absorption spectrometer. The control value of K⁺ content of the tissue was 54.8 μ equiv./g (see Table 1).

Addition to incubation medium	Water uptake (μ l/100mg initial wet wt.)	Na ⁺ content (μ equiv./g initial wet wt.)	K ⁺ content (μ equiv./g initial wet wt.)	K ⁺ efflux (decrease of K ⁺ content from that in the control) (μ equiv./g initial wet wt.)
None	43.7 $\pm$ 2.5	181.5 $\pm$ 0.6	20.9 $\pm$ 1.7	33.9
Tetrodotoxin	33.9 $\pm$ 3.8	169.0 $\pm$ 1.9	22.9 $\pm$ 2.9	31.9

Table 6. *Effects of 2,4-dinitrophenol and of high K⁺ concentration on the water uptake and on the concentration of ²²Na⁺ in incubated rat brain-cortex slices with or without tetrodotoxin (3 μ M)*

Experimental conditions were as in Table 2.

Substances added to incubation medium	O ₂ uptake (μ mol/100mg initial wet wt.)	Water uptake (μ l/100mg initial wet wt.)	²² Na ⁺ content (μ equiv./g initial wet wt.)
10mm-Glucose (control)	10.2 $\pm$ 0.2	17.7 $\pm$ 1.2	107.6 $\pm$ 1.0
10mm-Glucose + 30 μ M-2,4-dinitrophenol	16.6 $\pm$ 0.1	28.1 $\pm$ 2.1	143.2 $\pm$ 0.2
10mm-Glucose + 30 μ M-2,4-dinitrophenol + tetrodotoxin	17.4 $\pm$ 0.3	25.1 $\pm$ 3.3	140.3 $\pm$ 4.4
10mm-Glucose + 100mm-KCl	14.5 $\pm$ 0.2	38.0 $\pm$ 2.7	138.7 $\pm$ 4.8
10mm-Glucose + 100mm-KCl + tetrodotoxin	14.4 $\pm$ 0.3	37.7 $\pm$ 1.9	143.2 $\pm$ 3.0
10mm-Glucose + 27mm-KCl	12.3 $\pm$ 0.3	13.2 $\pm$ 2.4	104.1 $\pm$ 2.9
10mm-Glucose + 27mm-KCl + tetrodotoxin	11.6 $\pm$ 0.3	15.0 $\pm$ 2.1	106.6 $\pm$ 3.7
27mm-KCl; glucose absent	5.4 $\pm$ 0.2	43.4 $\pm$ 2.0	195.6 $\pm$ 3.2
27mm-KCl + tetrodotoxin; glucose absent	6.0 $\pm$ 0.1	44.9 $\pm$ 0.7	193.4 $\pm$ 0.3
10mm-Glucose + 27mm-KCl + 10 μ M-protoveratrine	13.6 $\pm$ 0.2	22.4 $\pm$ 1.0	123.5 $\pm$ 2.5
10mm-Glucose + 27mm-KCl + 10 μ M-protoveratrine + tetrodotoxin	11.2 $\pm$ 0.2	12.8 $\pm$ 1.6	108.4 $\pm$ 3.5

cortex slices from cat brain, with which Bourke & Tower (1966a,b) obtained a significant increase of water uptake (amounting to 15.9% above the control value) in the presence of 27mm-potassium chloride.

Examination of the cation content of the brain tissue showed that after incubation for 1 h in the presence of added 27mm-potassium chloride the content of K⁺ was 90.4 μ equiv./g. As the Na⁺ content was 104 μ equiv./g (Table 6), the total Na⁺ and K⁺ was 194 μ equiv./g. The concentration of

Na⁺ and K⁺ in the tissue was $\frac{194}{0.932} = 201$ mequiv./l,

which is higher by 21 mequiv./l than the external concentration of Na⁺ and K⁺ (180 mequiv./l). This value is but little different from that (15 mequiv./l) found in the absence of added K⁺ (Table 1). Thus in the presence of an external added concentration of 27mm-K⁺ an influx of K⁺ takes place to maintain

iso-osmoticity, with no increased water uptake by the tissue. The addition of tetrodotoxin (3 μ M) does not affect these values.

When 27mm-potassium chloride is added to the incubation medium devoid of glucose there is an increased influx of ²²Na⁺ by the slices (Table 6) that exceeds that found in the absence of the additional quantity of potassium chloride (Table 4). Tetrodotoxin, however, has no depressing effect on this influx of ²²Na⁺ (Table 5), in contrast with its definite effect when present in the normal medium containing 5mm-potassium chloride (Table 4). Moreover the partial suppression of the increased water uptake brought about by tetrodotoxin in an incubation medium devoid of glucose (Table 4) does not take place when 27mm-potassium chloride is added to this medium (Table 6).

Addition of 27mm-potassium chloride to the glucose-containing incubation medium in the

presence of protoveratrine ($10\mu\text{M}$) results in an increased influx of $^{22}\text{Na}^+$, rather less than that found in the absence of the additional K^+ (Tables 4 and 6). The presence of tetrodotoxin ($3\mu\text{M}$) still brings about a significant decrease in the influx of $^{22}\text{Na}^+$ in the presence of 27 mM-potassium chloride amounting to $15.1\mu\text{equiv./g}$ (Table 6). This may be compared with the fall in influx of $^{22}\text{Na}^+$, in the absence of the additional quantity of K^+ , amounting to $29.8\mu\text{equiv./g}$. It is therefore evident that 27 mM- K^+ diminishes the effect of tetrodotoxin in the presence of protoveratrine in incubated brain-cortex slices even if it does not abolish it. A similar partial suppressing action of 27 mM-potassium chloride on the effect of tetrodotoxin is seen in the respiratory rate and in the increased water uptake (Tables 4 and 6).

Effects of tetrodotoxin on the caudate nucleus

Similar experiments were carried out on the caudate nuclei of adult rat brains. Caudate nuclei were chosen for experiment as they are relatively poor in glia.

Results given in Table 7 show that the presence of ouabain (0.1 mM) brings about an increased influx of $^{22}\text{Na}^+$ that is partly suppressed by the addition of tetrodotoxin ($3\mu\text{M}$). It is therefore evident that, as with cortex slices, the ouabain-stimulated Na^+ influx into caudate nuclei has a tetrodotoxin-sensitive component.

However, in contrast with cortex slices, caudate nuclei show no significant increase of water uptake in the presence of ouabain, and the water uptake is not affected by tetrodotoxin (Table 7).

Effects of tetrodotoxin on the uptake of water and of $^{22}\text{Na}^+$ by brain-cortex slices from 20-day-old rats

Results given in Table 8 show that the effect of ouabain in increasing the influx of $^{22}\text{Na}^+$ and the water uptake of incubated brain-cortex slices from

20-day-old rats resembles quantitatively the effects found with slices from adult rats (Table 4). Moreover tetrodotoxin exerts diminishing effects on both the influx of $^{22}\text{Na}^+$ and the water uptake (Table 8) in a manner similar to that found with slices from more adult rats (Table 4).

The effect of omitting glucose from the incubation medium on the water uptake of incubated cortex slices from 20-day-old rats is not as marked as that found with slices from adult rats, but tetrodotoxin exerts a similar retarding effect (Tables 8 and 4). The increase in influx of $^{22}\text{Na}^+$ due to deprivation of glucose is similar to that found with slices from adult rats and the blocking effect of tetrodotoxin on this process is quite evident (Table 8).

DISCUSSION

Results recorded above (Table 1) show that under a variety of conditions (presence of ouabain or protoveratrine or absence of glucose) the changes of water uptake brought about in rat brain-cortex slices are identical, within the experimental error of these investigations, with those calculated for the maintenance of iso-osmoticity from the changes in tissue concentrations of Na^+ and K^+ . A similar conclusion applies also to brain-cortex slices from guinea pigs incubated in a glucose-bicarbonate medium containing 5 mM-L-glutamate (Harvey & McIlwain, 1968). Even when the external concentration of K^+ is increased by 27 mequiv./l K^+ flows into the isolated rat brain tissue to achieve iso-osmoticity without significant effect on the content of Na^+ or on the water uptake (Table 6).

The finding, described in the preceding paper (Okamoto & Quastel, 1970), that a proportionality exists between the water uptake of incubated rat brain slices and the ATP concentration suggests that much of the water uptake is intracellular. The extracellular space, which presumably includes the narrow clefts that separate neurons and glia and which serve as channels through which Na^+

Table 7. *Effects of tetrodotoxin on the uptake of water and of $^{22}\text{Na}^+$ by the caudate nucleus in the presence of ouabain*

The caudate nucleus was dissected from adult rat brain and cut into three pieces. Six such pieces (average wet wt. 42 mg) were incubated in 1.1 ml of Krebs-Ringer phosphate medium containing 10 mM-glucose in O_2 at 37°C for 1 h. $^{22}\text{Na}^+$ ($0.33\mu\text{Ci/ml}$ of medium) was tipped from the side tube of the manometric vessel into the medium after 5 min incubation. Tetrodotoxin ($3\mu\text{M}$) was added to the medium at the commencement of the experiment.

Substances added to the incubation medium	O_2 uptake	Water uptake	$^{22}\text{Na}^+$ content
	($\mu\text{mol}/100\text{mg}$ initial wet wt.)	($\mu\text{l}/100\text{mg}$ initial wet wt.)	($\mu\text{equiv./g}$ initial wet wt.)
10 mM-Glucose (control)	6.7 ± 0.7	21.5 ± 2.2	134.7 ± 0.8
10 mM-Glucose + 0.1 mM-ouabain	6.5 ± 0.9	22.5 ± 2.4	153.5 ± 2.9
10 mM-Glucose + 0.1 mM-ouabain + tetrodotoxin	5.8 ± 0.6	21.3 ± 3.0	141.1 ± 3.4

Table 8. *Effects of tetrodotoxin on the uptake of water and $^{22}\text{Na}^+$ under different incubation conditions by brain-cortex slices from 20-day-old rats*

Incubation was carried out for 1 h in O_2 at 37°C in Krebs-Ringer phosphate medium containing 10 mM-glucose except where otherwise stated. $^{22}\text{Na}^+$ (0.33 $\mu\text{Ci}/\text{ml}$ of medium) was tipped from the side tube of the manometric vessel into the medium 5 min after incubation had commenced. Tetrodotoxin (3 μM) was added to the medium at the commencement of the experiment.

Substances added to the incubation medium	O_2 uptake ($\mu\text{mol}/100\text{mg}$ initial wet wt.)	Water uptake ($\mu\text{l}/100\text{mg}$ initial wet wt.)	$^{22}\text{Na}^+$ content ($\mu\text{equiv.}/\text{g.}$ initial wet wt.)
10 mM-Glucose (control)	8.5 ± 0.5	12.2 ± 1.5	116.4 ± 3.7
10 mM-Glucose + 0.1 mM-ouabain	7.6 ± 0.2	36.9 ± 0.4	186.5 ± 1.4
10 mM-Glucose + 0.1 mM-ouabain + tetrodotoxin	7.7 ± 0.2	23.1 ± 2.5	150.1 ± 2.7
Glucose absent	5.9 ± 0.2	32.4 ± 2.0	179.4 ± 3.0
Tetrodotoxin; glucose absent	5.7 ± 0.2	21.9 ± 3.4	153.8 ± 5.0

and K^+ as well as other substances move rapidly through the nervous system (Kuffler & Nicholls, 1966), does not appear to exceed 20% of the total volume (Van Harreveld, 1966; Rose, 1969). It may therefore be concluded that the major changes in concentrations of Na^+ and K^+ that occur under most of the various incubation conditions described above take place intracellularly, i.e. in the neurons and in the glia.

The weight of evidence indicates that the changes of water uptake *in vitro* due to various incubation conditions do not occur to an appreciable extent in the neurons. Zadunaisky, Wald & De Robertis (1965) conclude that the gain in water of isolated incubated brain tissue is due to retention of salts largely by glial cells. Electron-microscopic studies (Gerschenfeld, Wald, Zadunaisky & De Robertis, 1959) showed that no swelling of neurons occurs in incubated brain slices, although there is marked swelling of the astrocytes. Studies of the brain cortex of developing kittens (Tower & Bourke, 1966; Tower, 1967) make it probable that mature cortical glial cells are largely associated with the changes of water uptake found *in vitro*.

Our experimental results obtained with the caudate nuclei containing relatively few glial cells show that the presence of ouabain exerts no promoting effect on the water uptake (Table 7), in contrast with its effect on brain-cortex slices (Table 1), although an increased Na^+ influx takes place. This fact supports the conclusion that the major changes in water uptake *in vitro*, under our experimental conditions, occur in the glia.

It follows that the net influx of ions, which is responsible for the increased water uptake *in vitro*, probably takes place in the glia. The lack of swelling in the cortical neurons in tissue slices, shown by the electron microscope, indicates that neuronal influx of Na^+ is accompanied by an equal efflux of neuronal K^+ during the experimental period of incubation.

Most of the experiments on Na^+ influx described above have been carried out with $^{22}\text{Na}^+$. This procedure is satisfactory because the results given in Table 2 show that the content of $^{22}\text{Na}^+$, under our experimental conditions, is an accurate representation of the total Na^+ present in the brain tissue slices at the termination of the incubation period.

The results recorded in these studies of the influx of $^{22}\text{Na}^+$ and of the water uptake by adult slices show that (a) the increased influx of $^{22}\text{Na}^+$ due to the presence of ouabain or protoveratrine, or to the application of electrical impulses, or to the absence of glucose, has a tetrodotoxin-sensitive component, and (b) the increased water uptake found on incubation of the tissue in the presence of ouabain or in the absence of glucose or on application of electrical pulses also has a tetrodotoxin-sensitive component.

These results may be understood without resort to any assumption of the action of tetrodotoxin other than its known effect on the sodium-current system responsible for the generation of action potentials at neuronal membranes (Kao, 1966). It is, however, necessary to suppose that action potentials do in fact occur in brain slices *in vitro*, under our experimental conditions, unless tetrodotoxin is present. It is well known that depolarization of neurons *in vivo* causes them to generate action potentials (Granit, 1964). It seems not unlikely, under our experimental conditions, that as the neurons depolarize due to loss of K^+ , with block of adenosine triphosphatase, there is a period during which action potentials are generated or when there is at least activation of the Na^+ -current system on which tetrodotoxin acts.

As it is known that glia are not electrically excitable cells and do not generate action potentials (Kuffler & Nicholls, 1966; Kuffler, 1967), it is evident that the effect of tetrodotoxin, in diminishing influx of $^{22}\text{Na}^+$ into incubated brain tissue, is confined to the neurons. The problem, therefore,

arises as to how tetrodotoxin, by diminishing cation influx into the neurons, on incubation under various conditions brings about a diminution of water uptake into the glia. The most obvious explanation is that the K^+ that comes out from the neurons when the Na^+ goes in, in association with an action potential, is largely taken up by the glia together with Cl^- , causing increased water uptake. Tetrodotoxin, by preventing action potential generation and hence increased efflux of neuronal K^+ , suppresses the increased uptake of K^+ by the glia and thereby the increased water uptake of the tissue. Such a water influx into glia is required to maintain iso-osmoticity of the glial cells, if a Donnan equilibrium is maintained, with consequent influx of K^+ and Cl^- , when the K^+ concentration is increased and the Na^+ concentration is decreased in the extracellular space after an action potential. Thus the major consequence of combined K^+ and Cl^- permeability of the glial cells will be a buffering of the extracellular K^+ concentration. This may indeed be considered an important function of the glial cells.

The results given in Tables 3 and 4 show that, on incubation of brain tissue in the presence of ouabain or of protoveratrine or in the absence of glucose or on application of electrical impulses, there is a correspondence between the diminished $^{22}Na^+$ influx due to tetrodotoxin and the accompanying diminished cation uptake into the glia, equivalent, for maintenance of iso-osmoticity, to the diminished water uptake. It therefore appears that suppression of efflux of K^+ from neurons by tetrodotoxin, under the given experimental conditions, is accompanied by suppression of influx of K^+ into the glia.

The results also support the conclusion (Kuffler, 1967; Kuffler & Nicholls, 1966; Trachtenberg & Pollen, 1970) that glial cells are selectively permeable to K^+ , and as a consequence of the release of K^+ by neighbouring neurons and its transient accumulation in the intracellular clefts the membrane of the glial cell becomes depolarized. As the K^+ disappears by uptake and diffusion the glial potential returns to normal (Kuffler, 1967). It has been suggested (Trachtenberg & Pollen, 1970) that when accumulations of K^+ are released during intensive and prolonged neural activity flow of K^+ into neighbouring glial cells is a mechanism of K^+ redistribution.

Results given in Table 6 show that, in the presence of increased K^+ (100 mequiv./l) in the incubation medium, when there is increased influx of $^{22}Na^+$ into the isolated brain tissue and increased water uptake, the addition of tetrodotoxin ($3 \mu M$) brings about no significant changes. This is to be expected for the large depolarization due to such a high K^+ concentration (Kuffler, 1967; Gibson &

McIlwain, 1965) should prevent the generation of action potentials (Hodgkin & Huxley, 1952) and therefore occlude any effect of tetrodotoxin. The development of action potentials during incubation in the absence of glucose, enabling tetrodotoxin to exercise inhibitory effects on the increased uptake of $^{22}Na^+$ or of water (Table 4), is evidently suppressed by the presence of 27 mM-potassium chloride in the incubation medium. Under such conditions tetrodotoxin has no effect (Table 6). However, the development of action potentials during incubation with protoveratrine ($10 \mu M$), shown by the inhibitory effects of tetrodotoxin on the increased $^{22}Na^+$ and water uptake (Table 4), is not completely suppressed by 27 mM-potassium chloride. Under such conditions tetrodotoxin still exerts some inhibitory activity (Table 6).

When incubation of the slices is carried out in presence of $30 \mu M$ -2,4-dinitrophenol there appears to be no generation of action potentials, for although there occurs increased uptake of $^{22}Na^+$ or of water there is no effect of tetrodotoxin on these uptakes under the given experimental conditions.

It is evident that the generation of action potentials during incubation of slices in the presence of ouabain, or in the absence of glucose, may occur with 20-day-old rats, as well as with more mature rats, for the presence of tetrodotoxin exerts marked suppressive effects on the increased uptake of $^{22}Na^+$ and of water (Table 8). The effects of tetrodotoxin on the uptake of $^{22}Na^+$, under these conditions, seem to be somewhat larger with brain-cortex slices of 20-day-old rats than those with brain-cortex slices of more mature animals. This may be correlated with the fact that the proportion of neurons in the total brain cell population tends to diminish with increasing age (Brizze, Vogt & Kharechka, 1964).

The results of experiments with L-glutamate (Table 4) appear to indicate that this substance presents an exception to the rule that when tetrodotoxin diminishes Na^+ influx there is a corresponding diminution of water uptake. This result suggests that the action of L-glutamate is not restricted to neurones.

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ADDENDUM

Calculations of ionic fluxes

On the assumptions that tetrodotoxin acts only on the neurons, that neuronal Na^+ influx and neuronal K^+ efflux are approximately equal under the given experimental conditions and that in-

creased water uptake in the tissue slices due to changed incubation conditions takes place largely in the glia, it is possible to make certain calculations on the extents of fluxes of Na^+ and K^+ in the neurons and glia of the tissue slices.

An example of such a calculation is as follows.

Table 1. *Calculations of changes of ionic contents in incubated rat brain-cortex slices under various incubation conditions from observations of $^{22}\text{Na}^+$ and of water uptake (Tables 3 and 4 of main paper)*

Control value of Na^+ content is $107.6 \mu\text{equiv./g}$ (Table 4 of main paper). For details of calculations see the text. All values are given in terms of g of tissue.

Incubation condition	Observed increase of Na^+ content in the tissue above that in the control ($\mu\text{equiv./g}$)	Calculated decrease of K^+ content in the tissue ($\mu\text{equiv./g}$)	Calculated increase of K^+ content of glia due to action potentials ($\mu\text{equiv./g}$)
Ouabain	59.4	23.9	—
Ouabain + tetrodotoxin	33.1	21.9	—
Difference due to suppression of action potential	26.3	2.0	24.3
Protoveratrine	27.0	15.2	—
Protoveratrine + tetrodotoxin	-2.8	4.5	—
Difference due to suppression of action potential	29.8	10.7	19.1
Absence of glucose	74.8	35.0	—
Absence of glucose + tetrodotoxin	60.6	35.8	—
Difference due to suppression of action potential	14.2	-0.8	15.0
Electrical pulses	19.4	8.8	—
Electrical pulses + tetrodotoxin	4.1	5.9	—
Difference due to suppression of action potential	15.3	2.9	12.4

In the presence of ouabain there is an increased content of Na^+ in the tissue due to the combined effects of the suppression of the sodium pump (inactivation of $\text{Na}^+ + \text{K}^+$ -stimulated adenosine triphosphatase) and the presumptive generation of action potentials. This amounts to $59.4 \mu\text{equiv./g}$ (Table 4 of main paper). In the presence of both ouabain and tetrodotoxin the increased influx of Na^+ is due only to the suppression of the sodium pump. This amounts to $33.1 \mu\text{equiv./g}$ (Table 4 of main paper). Therefore $59.4 - 33.1 = 26.3 \mu\text{equiv./g}$ represents the Na^+ influx in the neuron due to generation of the action potential. (It is assumed that ouabain and tetrodotoxin do not interfere with each other's activities at the concentrations used.)

In the presence of ouabain the increased water uptake is $232 \mu\text{l/g}$, which is equivalent, for iso-osmoticity, to a cation uptake of $232 \times 0.153 = 35.5 \mu\text{equiv./g}$. As the increased Na^+ influx into the tissue is $59.4 \mu\text{equiv./g}$, it follows that the increased K^+ efflux from the tissue is $59.4 - 35.5 =$

$23.9 \mu\text{equiv./g}$. In the presence of ouabain and tetrodotoxin the increased water uptake is $73 \mu\text{l/g}$, which is equivalent, for iso-osmoticity, to a cation uptake of $73 \times 0.153 = 11.2 \mu\text{equiv./g}$. Since the increased Na^+ uptake is $33.1 \mu\text{equiv./g}$ the increased K^+ efflux is $33.1 - 11.2 = 21.9 \mu\text{equiv./g}$.

It is therefore evident that when the generation of action potentials in the presence of ouabain is suppressed by tetrodotoxin there is a resultant decreased influx of Na^+ amounting to $26.3 \mu\text{equiv./g}$, and the net change of efflux of K^+ from the tissue is only $23.9 - 21.9 = 2.0 \mu\text{equiv./g}$. However, for influx of Na^+ into the neuron of $26.3 \mu\text{equiv./g}$ there is an efflux of $26.3 \mu\text{equiv. of } \text{K}^+/\text{g}$ from the neuron. Since the net change of efflux of K^+ from the tissue due to abolition of the action potentials is $2.0 \mu\text{equiv./g}$ it follows that $26.3 - 2.0 = 24.3 \mu\text{equiv./g}$ is retained in the tissue and is therefore taken up by the glia. The results of calculations of this nature are shown in Table 1.