Effects of Tetrodotoxin and Anaesthetics on Brain Metabolism and Transport During Anoxia

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1. Tetrodotoxin, at concentrations at which it abolishes generation of action potentials in the nervous system, enhances by about 300% the rate of anaerobic glycolysis of brain-cortex slices from adult rats, or from adult and infant guinea pigs. This occurs to a greater extent in Ca^{2+} -deficient incubation media than in Ca^{2+} -rich media. Tetrodotoxin has no accelerative effect on cerebral aerobic glycolysis. 2. Tetrodotoxin does not affect the rate of anaerobic glycolysis of 2-day-old rat brain-cortex slices, nor that of adult rat kidney medulla, nor that of an extract of an acetone-dried powder of brain. 3. Tetrodotoxin does not affect the rate of penetration of glucose into brain slices. 4. Its effect is not apparent if it is added 10 min or later after the onset of anoxia. 5. Its effect diminishes as the concentration of K^+ in the incubation medium is increased while that of Na^+ is decreased. 6. Its salient effect, at the onset of anoxia, is to diminish influx of Na^+ into, and efflux of K^+ from, the brain slices. 7. Substances that promote cerebral influx of Na^+ , e.g. protoveratrine, sodium L-glutamate, diminish the accelerative action of tetrodotoxin. 8. It is concluded that tetrodotoxin exerts its effect on anaerobic glycolysis by suppressing, at the onset of anoxia, the generation of action potentials and thereby the accompanying influx of Na^+ and efflux of K^+ . It is suggested that glycolytic stimulation occurs because a rate-limiting step, e.g. operation of pyruvate kinase, is stimulated by K^+ and depressed by Na^+ . 9. Local anaesthetics behave in a manner similar to that of tetrodotoxin in enhancing cerebral anaerobic glycolysis. 10. Sodium Amytal has a marked effect at relatively high concentration. 11. Tetrodotoxin diminishes efflux of amino acids, particularly glutamate and aspartate, at the onset of anoxia.

It has been shown (Adams & Quastel, 1956) that a variety of organic bases, e.g. pyrrole, quinoline, *p*-chloroaniline, accelerate the rate of anaerobic glycolysis of guinea-pig brain-cortex slices in a Ca^{2+} -free medium containing only Na^+ and K^+ as cations. It was also found that such bases exert neurological effects, simulating those of Ca^{2+} ions, in abolishing the spontaneous firing of cat cervical sympathetic ganglia. It was suggested that these bases act at the brain cell membrane, establishing there new cationic equilibria which are reflected, on the one hand, by changed rates of glucose breakdown in the neurone and, on the other hand, by changed neurophysiological activities.

More recently it has been found that tetrodotoxin, a neurological poison of great potency, inhibits the increased respiration of rat brain-cortex slices that occurs on the application of electrical pulses or in the presence of protoveratrine or when Ca^{2+} ions are omitted from the medium. Moreover, it has been shown that the inhibition of cerebral acetate oxidation, owing to the influx of Na^+ , that occurs when electrical pulses are applied to rat brain-cortex slices, is completely blocked by small concentrations of

tetrodotoxin (Chan & Quastel, 1967, 1970; Okamoto & Quastel, 1970). It was concluded that tetrodotoxin brings about its potent metabolic effects by blocking the influx of Na^+ that occurs on electrical stimulation or in the presence of protoveratrine. McIlwain (1967) has found similarly that tetrodotoxin inhibits the respiratory response induced by electrical stimulation of guinea-pig brain-cortex slices and that the K^+ content of the electrically stimulated slices is enhanced by the presence of tetrodotoxin.

Tetrodotoxin is well known to abolish the generation of action potentials and the associated influx of Na^+ at the neuronal membrane (Kao, 1966; Narahashi *et al.*, 1964, 1966; Nakamura *et al.*, 1965; Moore & Narahashi, 1967). The demonstration that tetrodotoxin prevents the influx of Na^+ in rat brain-cortex slices incubated under various metabolic conditions, e.g. absence of glucose from the incubation medium, the presence of protoveratrine or of ouabain, led to the conclusion that action potentials may be generated in brain-cortex slices under a variety of incubation conditions (Okamoto & Quastel, 1970).

We have found that tetrodotoxin exerts a powerful stimulating effect on the rate of anaerobic glycolysis

in brain-cortex slices from rats or guinea pigs, especially when incubated in Ca^{2+} -free media. The effect is reminiscent of that found with pyrrole etc., but the effect is far greater in magnitude and takes place at much smaller concentrations. Moreover, we have found that local anaesthetics, at pharmacologically active concentrations, behave in a manner similar to that of tetrodotoxin.

We have investigated this effect of tetrodotoxin in some detail, not only to throw light on its mode of action, but to obtain further understanding of the processes taking place at the brain cell membrane at the onset of anoxia. The results of this investigation, and their possible implications, are described below.

Materials

Animals

Adult rats of the Wistar strain, weighing 200–250 g, and adult male guinea pigs (weighing 300–350 g), newly born guinea pigs of English short-hair strain, and infant rats (Wistar, 1–3 days old, both sexes) were obtained from the Department of Zoology Vivarium, University of British Columbia, or from the Animal Unit, Faculty of Medicine, University of British Columbia. All the adult animals had free access to food and water, but the infant animals were separated from their mothers a few hours before the start of the experiments.

Enzymes

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (type VI, from yeast) and hexokinase (EC 2.7.1.1) (type C-300, from yeast) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Lactate dehydrogenase (EC 1.1.1.27) (from rabbit muscle) was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Chemicals

All common laboratory chemicals were of reagent grade and were used without further purification. Lithium lactate, NADP^+ and tetrodotoxin were obtained from Calbiochem. Protoveratrine was obtained from K & K Laboratories, Plainview, N.Y., U.S.A.; sodium Amytal from E. Lilly and Co., Montreal, P.Q., Canada, and procaine and lidocaine from the Department of Pharmacology, University of British Columbia. $[2\text{-}^{14}\text{C}]$ Glucose was obtained from The Radiochemical Centre, Amersham, U.K., and $[2\text{-}^{14}\text{C}]$ glycine and sodium $[\text{U-}^{14}\text{C}]$ glutamate were obtained from Volk Radiochemical Co., Chicago, Ill., U.S.A. EGTA [ethanedioxybis(ethylamine)-tetra-acetate] and EDTA were obtained from Sigma Chemical Co.

Methods

Preparation of brain-cortex slices

Rats were killed by stunning, the brains removed and cerebral-cortex slices (about 0.4 mm thick) were prepared with a Stadie–Riggs tissue slicer moistened with the glucose-containing medium to be used for subsequent incubation. One dorsal and one lateral slice ('first slices') (total, 60–70 mg wet wt.) from the same brain were used. Guinea-pig brain-cortex slices were prepared in a similar manner, except that, for lateral slices, parietal lobes were first cut out before slicing and only one slice (40–55 mg wet wt.) was used in each experiment. Infant rats were decapitated, their brains removed and one slice was cut from the temporal–parietal portion of each hemisphere. Two slices from each brain (70–90 mg wet wt.) were used in each experiment. Brain-cortex slices from newly born guinea pigs were prepared in the same way as that used for the older animals.

Incubation procedure

The slices were weighed quickly after removal of adherent fluid by touching with filter paper, and suspended in 3 ml of precooled incubation medium (whose composition is given in the Tables) contained in a conventional Warburg manometric vessel. Additions to the incubation medium were made either at the beginning of the experiment or during the course of incubation by tipping from the side arm of the vessel. Incubations were carried out at 37°C in an atmosphere of $\text{N}_2 + \text{CO}_2$ (95:5) or of $\text{O}_2 + \text{CO}_2$ (95:5) as indicated in the tables. In some experiments the gas phase was changed during the course of incubation. The incubation period was usually 1 h except where indicated.

Incubation media

Two types of incubation media were used. (a) Krebs–Ringer bicarbonate medium of the following composition: 119 mM-NaCl, 5 mM-KCl, 3.6 mM- CaCl_2 , 1.2 mM- MgSO_4 , 25 mM- NaHCO_3 . (b) Ca^{2+} -free Locke–bicarbonate medium of the following composition: 120 mM-NaCl, 5 mM-KCl, 29 mM- NaHCO_3 . This medium, free of Ca^{2+} and Mg^{2+} , as used by Adams & Quastel (1956), is useful for demonstrating the most marked effects of tetrodotoxin or of local anaesthetics on the rates of anaerobic glycolysis. Control experiments showed that the presence or absence of Mg^{2+} in the incubation medium makes very little difference to the results. Ca^{2+} , however, has marked effects that will be described below.

These incubation media were gassed with $\text{N}_2 + \text{CO}_2$ (95:5) before use.

Preparation of kidney medulla

Rats were stunned by a blow on the head, their kidneys quickly removed and each kidney was cut into two equal halves. After removal of the cortex, the two halves of the medulla (each 0.5–1 mm thick and weighing together 120–140 mg wet wt.) were used in each experiment.

Preparation of acetone-dried powder of brain

Extracts of acetone-dried powder of rat brain were prepared by the method of Harpur & Quastel (1949). Rat brain-cortex slices were ground in ice-cold acetone and, after filtering and washing with ice-cold acetone, the resulting powder was stored in a vacuum desiccator at 4°C until required. A portion (100 mg) of the acetone-dried powder was extracted with 10 ml of a solution containing 100 mM-nicotinamide, 33 mM-cysteine and 156 mM-NaCl. The extract was centrifuged and 1 ml of the supernatant was used in each experiment.

Measurements of the rate of glycolysis

Manometric. This method was most frequently used in anaerobic experiments and control experiments showed that the results were identical with those obtained by the enzymic method described below. The course of anaerobic glycolysis was followed by measuring manometrically the rate of CO₂ evolution from the bicarbonate medium in the Warburg apparatus, at pH 7.4, in an atmosphere of N₂+CO₂ (95:5).

Enzymic. A portion of the incubation medium, after treatment with HClO₄ and subsequent neutralization (after centrifuging) with 5 M-K₂CO₃, was diluted fivefold with water and a 1.5 ml sample was used for enzymic assay of lactate, by using the lactate dehydrogenase system (Hohorst, 1963).

Measurements of amino acid uptake and efflux

Amino acid uptake. The uptake of [2-¹⁴C]glycine or of [U-¹⁴C]glutamate into incubated rat brain-cortex slices was measured by methods described in detail by Nakazawa & Quastel (1968). The incubations were carried out at 37°C for 15 min in a Ca²⁺-free Locke-bicarbonate medium, containing 20 mM-glucose, in an atmosphere of N₂+CO₂ (95:5). Labelled amino acids, together with non-radioactive carrier amino acids, were added from the side arm of the manometric vessel to the main vessel after attainment of temperature equilibrium. The radioactivity added to each vessel was 214 000 c.p.m. for [¹⁴C]glutamate and 145 000 c.p.m. for [¹⁴C]glycine. Amino acid uptakes were calculated as $\mu\text{mol/g}$ initial wet wt. of tissue by dividing the radioactivity of the incubated slices, expressed as c.p.m./g wet wt., by the specific

radioactivities (c.p.m./ μmol) of the amino acid present in the incubation medium.

Amino acid efflux. Incubation of rat brain-cortex slices in a Ca²⁺-free Locke-bicarbonate medium containing 20 mM-glucose was carried out at 37°C for 30 min in an atmosphere of N₂+CO₂ (95:5). At the end of the incubation period, the slices were homogenized in 2.5 ml of 5% (w/v) cold trichloroacetic acid. The homogenate was left in the cold for 30 min and centrifuged. The supernatant was transferred to another tube. The precipitate was washed with 1 ml of cold 5% trichloroacetic acid and the washings were added to the supernatant, which was then extracted with 3 $\times$ 3 ml of diethyl ether to remove trichloroacetic acid. Ether was removed from the water phase by blowing air through it, and the acid-free extract, after suitable dilution, was used for amino acid analysis with a Beckman model 120 B amino acid analyser.

Fig. 1. Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices

Rat brain-cortex slices were incubated anaerobically at 37°C with or without tetrodotoxin (2 μM) in Ca²⁺-free or Ca²⁺-containing media in the presence of 20 mM-glucose. ○, Ca²⁺-free Locke-bicarbonate medium; ▲, Krebs-Ringer-bicarbonate medium; ■, Krebs-Ringer-bicarbonate medium + tetrodotoxin; ●, Ca²⁺-free Locke-bicarbonate medium + tetrodotoxin.

Measurements of Na^+ and K^+

The incubated slices, after removal of adherent fluid by filter-paper strips, were homogenized in 3 ml of 5% trichloroacetic acid and left in the cold for 1 h. The supernatants obtained after centrifugation were diluted with water to obtain final cation concentrations of about 0.05–0.10 $\mu\text{equiv./ml}$ for both Na^+ and K^+ . Atomic absorptions were measured at 294.3 nm and 383.3 nm for Na^+ and K^+ respectively with a Perkin-Elmer model 303 atomic-absorption spectrophotometer. The contents of Na^+ and K^+ given as $\mu\text{equiv./g}$ initial wet wt. were calculated from standards run with each set of experiments.

Determination of ATP

ATP was determined by an enzymic method with hexokinase and glucose 6-phosphate dehydrogenase (Greengard, 1963). The tissue slices, at the appropriate time in the experiment, were quickly homogenized with 2 ml of 6% (w/v) HClO_4 , the homogenate left over ice for 30 min and the supernatant, after centrifuging, was neutralized with K_2CO_3 . NADPH was measured fluorimetrically (Itoh & Quastel, 1970). ATP concentration was calculated as that amount contained in 1 g initial wet wt. of brain tissue.

Reproducibility of results

Each experiment was carried out at least three times. Some variations in the absolute values were found with different batches of animals and so control experiments were always carried out in every set of experiments. Results below are the means of several values obtained in typical experiments and the standard deviations from the mean are given.

Results

Effects of tetrodotoxin on anaerobic glycolysis of rat brain slices

Results of typical experiments showing the acceleration due to tetrodotoxin (2 μM) on the course

of anaerobic breakdown of glucose into lactate by rat brain-cortex slices incubated in either a Ca^{2+} -containing or a Ca^{2+} -free medium are shown in Fig. 1. These results show also the enhancing effects caused by addition of Ca^{2+} , noted by Adams & Quastel (1956), and the fact that the stimulating action of tetrodotoxin is smaller in the presence of Ca^{2+} than in its absence. Similar effects of tetrodotoxin and of Ca^{2+} on the rate of anaerobic glycolysis were found with guinea-pig brain-cortex slices.

Effects of various concentrations of tetrodotoxin on anaerobic glycolysis of brain slices taken from animals of different ages

Tetrodotoxin had no significant effect on the anaerobic breakdown of glucose to lactate by slices of the brains of infant (2-day-old) rats. A marked effect occurred with 14-day-old rats. The brains of newly born guinea pigs responded to tetrodotoxin, so far as anaerobic glycolysis is concerned, as effectively as the adult animal brains. Results are shown in Table 1, which also demonstrates that marked effects of tetrodotoxin on cerebral anaerobic glycolysis in Ca^{2+} -free media occur at a tetrodotoxin concentration as low as 0.2 μM . We have preferred to use the concentration of 2 μM -tetrodotoxin for systematic work, though larger concentrations may bring about somewhat greater rates of anaerobic glycolysis (Table 1).

Effects of various glucose concentrations on tetrodotoxin-stimulated anaerobic glycolysis

Although the maximum rate of anaerobic glycolysis of rat brain-cortex slices in a Ca^{2+} -free medium occurred with a concentration of glucose at or somewhat below 5 mM, in the presence of 2 μM -tetrodotoxin the rate of glycolysis increased with glucose concentration until a maximum was obtained at about 50 mM. Typical results are shown in Fig. 2. Presumably this takes place because, in the presence of tetrodotoxin, the speed of glycolysis is so rapid that the rate of dif-

Table 1. Effects of different concentrations of tetrodotoxin on anaerobic glycolysis of brain-cortex slices

Cortex slices from rat or guinea-pig brain were incubated anaerobically at 37°C in a Ca^{2+} -free Locke-bicarbonate medium containing 20 mM-glucose. Tetrodotoxin, when present, was added at the start of the experiment. Lactate production was measured manometrically for a period of 1 h commencing 20 min after the start of the experiment.

Concn. of tetrodotoxin (μM)	Lactate formed ($\mu\text{mol/g}$ initial wet wt.)				
	2-day-old rat	14-day-old rat	Newly born guinea pig	Adult rat	Adult guinea pig
Nil	25.0 $\pm$ 1.5	25.4 $\pm$ 3.1	58.0 $\pm$ 5.4	20.0 $\pm$ 3.5	47.0 $\pm$ 5.6
0.2	28.6 $\pm$ 1.8	42.5 $\pm$ 1.3	133.0 $\pm$ 4.5	70.0 $\pm$ 6.4	80.0 $\pm$ 6.5
2.0	29.9 $\pm$ 5.9	48.3 $\pm$ 9.9	124.0 $\pm$ 5.5	85.0 $\pm$ 3.0	110.9 $\pm$ 10.3
10.0	29.8 $\pm$ 0.9	51.4 $\pm$ 6.6	126.0 $\pm$ 1.8	127.0 $\pm$ 6.5	139.1 $\pm$ 2.5

fusion of glucose into the brain cell becomes rate-limiting. In the absence of tetrodotoxin, the speed of glycolysis is so low as to make it independent of glucose concentrations in the incubation medium exceeding 5 mm.

Fig. 2. Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of various concentrations of glucose

Incubations were carried out anaerobically at 37°C in Ca^{2+} -free Locke-bicarbonate media with or without tetrodotoxin (2 μM). Measurements of lactate formed were made after anaerobic incubation for 20 min. ●, Tetrodotoxin absent; ○, tetrodotoxin present.

Effects of tetrodotoxin on the uptake of glucose by rat brain-cortex slices during anoxia

Experiments were carried out to observe whether tetrodotoxin accelerates the rate of diffusion of glucose into rat brain cells. The results of such experiments are shown in Table 2. The method adopted was to observe whether the anaerobic uptake of radioactivity from a Ca^{2+} -free incubation medium containing [2- ^{14}C]glucose (either 5 or 20 mm), during a short incubation period at 37°C, is affected by the presence of 2 μM -tetrodotoxin. It was expected that some of the accumulated radioactivity would be due to the formation of labelled lactate, which, however, diffuses very rapidly into the incubation medium. The results show that the presence of 2 μM -tetrodotoxin brings about very little acceleration, if any, of the rate of diffusion of glucose into the brain tissue. The small increase in uptake of radioactivity found is probably due to the formation of labelled lactate. To block the effects of glycolysis, experiments were carried out in the presence of sodium iodoacetate (0.2 mm), and Table 2 shows that iodoacetate does not significantly affect the diffusion of labelled glucose into the brain and that, in its presence, tetrodotoxin has no stimulating effect on the uptake of radioactivity from the medium.

Effects of tetrodotoxin on anaerobic glycolysis of rat kidney-medulla slices and of extracts of acetone-dried powder of brain

Tetrodotoxin (2 μM) exerted no effect on the rate of anaerobic glycolysis of rat kidney-medulla slices incubated in a Ca^{2+} -free saline medium containing 20 mm-glucose (Table 3), although the slices broke down glucose anaerobically at a rate comparable with that brought about by guinea-pig brain slices.

Table 2. Effects of tetrodotoxin on the uptake of glucose by rat brain-cortex slices during anoxia

Rat brain-cortex slices were incubated anaerobically at 37°C in a Ca^{2+} -free glucose-containing Locke-bicarbonate medium. Tetrodotoxin or iodoacetate or a mixture of these substances was added at the commencement of the experiment, i.e. at the onset of anoxia. After incubation for 15 min, 0.5 μCi of [2- ^{14}C]glucose was added (as described in the text), and incubation was carried out for a further 5 min. The tissue slices were then removed, rinsed rapidly and the radioactivity in them was measured as described in the text. The uptake of radioactivity was expressed in terms of glucose uptake by dividing the c.p.m./g found in the tissue by the specific radioactivity of the labelled glucose in the incubation medium.

Additions to the incubation medium Concn. of glucose in incubation medium ...	Glucose uptake ($\mu\text{mol/g}$ initial wet wt.)	
	5 mm	20 mm
Nil	3.16 ± 0.15	12.05 ± 0.6
Tetrodotoxin (2 μM)	3.48 ± 0.17	13.70 ± 0.7
Sodium iodoacetate (0.2 mm)	3.16 ± 0.15	12.80 ± 0.6
Tetrodotoxin (2 μM) + sodium iodoacetate (0.2 mm)	3.00 ± 0.15	11.90 ± 0.5

Table 3. *Effects of tetrodotoxin on anaerobic glycolysis of rat kidney-medulla slices and of acetone-dried powder extracts of rat brain*

Rat kidney-medulla slices were incubated anaerobically at 37°C in a Ca^{2+} -free Locke-bicarbonate medium containing 20mM-glucose. Tetrodotoxin, when present, was added at the commencement of the experiment, i.e. at the onset of anoxia. Acetone-dried powders of rat brain extracts, prepared as described in the text, were incubated anaerobically in a medium containing 52mequiv. of Na^+ /l, 28mequiv. of K^+ /l, 4mM- MgCl_2 , 33mM-cysteine, 33mM-nicotinamide, 0.5mM- NAD^+ and 0.8mM-ATP. Tetrodotoxin was added at the start of the experiment. Lactate production was measured manometrically over a period of 1 h commencing 20 min after the start of the experiment. Results are given for two successive 30 min periods.

Additions to the incubation medium	Lactate formed			
	In kidney-medulla slices ($\mu\text{mol/g}$ initial wet wt.)		In acetone-dried powder of rat brain ($\mu\text{mol/g}$ of powder)	
	First 30 min period	Second 30 min period	First 30 min period	Second 30 min period
None	41.0 $\pm$ 4.9	33.0 $\pm$ 1.8	410.0 $\pm$ 18.0	366.0 $\pm$ 18.0
Tetrodotoxin (2 μM)	43.7 $\pm$ 5.8	34.4 $\pm$ 7.1	434.0 $\pm$ 9.0	380.0 $\pm$ 4.0

Tetrodotoxin also exerted no significant stimulation of the anaerobic rate of glycolysis brought about by an extract of an acetone-dried powder of rat brain cortex. The results given in Table 3 show that the rate of lactate formation from glucose by an extract of acetone-dried powder of brain, examined under optimum conditions, amounts to 776 μmol of lactate/h per g of acetone-dried powder. This may be compared with the value of 127 μmol /h per g wet wt. of brain, or 635 μmol /h per g dry wt. of brain, found by using rat brain-cortex slices in the presence of 10 μM -tetrodotoxin (Tables 3 and 1). Therefore the rate of glucose conversion into lactate under anaerobic conditions, by an extract of an acetone-dried powder of rat brain incubated at 37°C in the presence of optimum quantities of NAD^+ and ATP, is of the same order of magnitude as the maximum rate of glycolysis found with rat brain-cortex slices incubated in a Ca^{2+} -free medium at 37°C in the presence of tetrodotoxin.

Effects of tetrodotoxin on aerobic glycolysis of rat brain slices

Tetrodotoxin (2 μM) had no stimulating effect on the rate of lactate formation from glucose by rat brain-cortex slices incubated aerobically at 37°C. A small inhibitory effect appeared to take place.

Effects of addition of tetrodotoxin at different time-intervals during anoxia on anaerobic glycolysis of rat brain slices

Results given in Fig. 3 show that when tetrodotoxin (2 μM) was added to rat brain-cortex slices incubated

Fig. 3. *Effects of addition of tetrodotoxin at different time-intervals during anoxia on anaerobic glycolysis of rat brain-cortex slices*

Incubations were carried out anaerobically at 37°C in Ca^{2+} -free Locke-bicarbonate medium containing 20mM-glucose. Measurements of lactate formed were made over a period of 1 h after the first 20 min of anaerobic incubation. Tetrodotoxin (2 μM) was added either at the start of the experiment, or after 2 min anaerobic incubation, or after 10 min anaerobic incubation. ■, Tetrodotoxin absent; □, tetrodotoxin added 10 min after start of anoxia; ●, tetrodotoxin added 2 min after start of anoxia; ○, tetrodotoxin added at start.

anaerobically in a Ca^{2+} -free medium 10 min after the onset of anoxia the accelerative effect of tetrodotoxin on anaerobic glycolysis disappears. The results show that tetrodotoxin was still effective if added 2 min after the onset of anoxia. Further experiments have shown that the tetrodotoxin effect is still present, although much decreased, at 5 min after anaerobiosis has commenced. It is therefore necessary, for observation of the effects of tetrodotoxin, to add it to the incubation medium at or before the time that anoxic conditions prevail.

Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of 1 mM-sodium pyruvate

In view of the well-known fact that pyruvate stimulates the rate of anaerobic glycolysis of brain slices, and in view of the possibility that tetrodotoxin may bring about its effects indirectly by causing changes in the brain cell concentration of pyruvate, experiments were carried out to observe whether tetrodotoxin will still exert its accelerative action on cerebral anaerobic glycolysis in the presence of an excess of pyruvate. The results given in Table 4 show that, in a Ca^{2+} -free medium, tetrodotoxin ($2\mu\text{M}$) exerted marked stimulating effects on the rate of anaerobic glycolysis of rat brain-cortex slices even in the presence of 1 mM- or 10 mM-sodium pyruvate. These results show that the enhancing effect of tetrodotoxin cannot be due to possible changes in the cell concentration of pyruvate. In a Ca^{2+} -containing (Krebs-Ringer) medium, containing 1 mM-sodium pyruvate, the stimulating action of tetrodotoxin is not so marked. This is because the presence of sodium pyruvate alone, in such a medium, brings about a

very high rate of anaerobic glycolysis, approaching the maximum attainable.

Effects of tetrodotoxin on anaerobic glycolysis and ATP contents of rat brain-cortex slices in the presence or absence of 2,4-dinitrophenol (0.1 mM) or of sodium pyruvate (1 mM)

Experiments were carried out to decide whether tetrodotoxin might exert its stimulating effect on cerebral anaerobic glycolysis by bringing about a change in the cell concentration of ATP. To test this point of view, rat brain-cortex slices were incubated in a Ca^{2+} -free glucose-containing saline medium in the presence or absence of 0.1 mM-2,4-dinitrophenol for 15 min anaerobically [in an atmosphere of $\text{N}_2 + \text{CO}_2$ (95:5)] succeeded by a period of 10 min aerobically [in an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5)]. At the end of the aerobic period, the ATP concentrations were measured. As expected, the ATP concentrations in the vessels containing 2,4-dinitrophenol were smaller than those in the control vessels. The aerobic period was then succeeded by a 1 h anaerobic period in which measurements of the lactate formed were made. In certain vessels, sodium pyruvate (1 mM) was present from the start of the experiment, glucose being added at the start of the second incubation period. This experiment was done to ensure that the pyruvate concentration in the tissue could not be considered a rate-limiting factor. Tetrodotoxin ($2\mu\text{M}$), when present, was added at the beginning of the experiment. Results in Table 5 show that tetrodotoxin does not affect the cell concentrations of ATP, in either the presence or the absence of 2,4-dinitrophenol, that are found at the onset of anoxia. They also show that tetrodotoxin exerts its stimulating

Table 4. *Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of 1 mM-sodium pyruvate*

Rat brain-cortex slices were incubated anaerobically at 37°C in Krebs-Ringer bicarbonate medium or in Ca^{2+} -free Locke-bicarbonate medium and in the presence or absence of tetrodotoxin and 1 mM-sodium pyruvate. Glucose (20 mM) was always present. The amount of lactate produced was estimated from the rates of release of CO_2 , measured manometrically as described in the text, for an incubation period of 1 h commencing 20 min after the start of incubation.

Additions to the incubation medium	Lactate formed ($\mu\text{mol/g}$ initial wet wt.)	
	Krebs-Ringer-bicarbonate medium	Ca^{2+} -free Locke-bicarbonate medium
Nil	38.0 ± 4.4	31.7 ± 4.3
Tetrodotoxin ($2\mu\text{M}$)	65.0 ± 5.0	76.0 ± 5.0
Sodium pyruvate (1 mM)	113.4 ± 6.3	75.0 ± 14.9
Tetrodotoxin ($2\mu\text{M}$) + sodium pyruvate (1 mM)	124.1 ± 5.4	117.0 ± 14.3
Sodium pyruvate (10 mM)		70.1 ± 12.7
Tetrodotoxin ($2\mu\text{M}$) + sodium pyruvate (10 mM)		128.1 ± 6.5

Table 5. *Effects of tetrodotoxin on anaerobic glycolysis and ATP contents of rat brain-cortex slices in the presence or absence of 2,4-dinitrophenol (0.1 mM) or of sodium pyruvate (1 mM)*

Rat brain-cortex slices were incubated at 37°C in a Ca²⁺-free Locke-bicarbonate medium containing 20mM-glucose, anaerobically for 15min and then aerobically for 10min. The contents of ATP in the tissue slices were measured at this time. The incubation mixture was then made anaerobic and incubation continued for a total of 100min. Lactate formation was measured manometrically for a period of 1h commencing 15min after the start of the second anaerobic incubation period. In one set of experiments, in which pyruvate was present from the start, glucose was added only at the beginning of the second anaerobic period.

Additions to the incubation medium	ATP content at the start of the second incubation period (μmol/g)	Lactate formed in 1 h in the second incubation period (μmol/g)	Sodium pyruvate (1 mM) present; glucose (20mM) added at the start of the second incubation period
			Lactate formed (μmol/g)
Nil	1.48	37.9 ± 1.8	79.5 ± 8.0
2,4-Dinitrophenol (0.1 mM)	1.16	26.8 ± 1.3	49.6 ± 7.1
Tetrodotoxin (2 μM)	1.42	146.0 ± 1.3	159.8 ± 6.7
2,4-Dinitrophenol (0.1 mM) + tetrodotoxin (2 μM)	1.17	117.0 ± 6.3	101.0 ± 10.9

Table 6. *Effects of time of addition of glucose on the accelerative effect of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices*

Rat brain-cortex slices were incubated anaerobically at 37°C in a Ca²⁺-free Locke-bicarbonate medium. Glucose (20mM) was added either at the start of the experiment or 15min after onset of anoxia. Lactate formation was measured manometrically for a period of 1h commencing 20min after addition of glucose.

Addition to the incubation medium	Lactate formed (μmol/g initial wet wt.)	
	Glucose added at start	Glucose added 15 min after onset of anoxia
Nil	29.0 ± 4.9	17.6 ± 1.8
Tetrodotoxin (2 μM)	83.5 ± 10.7	16.5 ± 4.0

effect on anaerobic glycolysis equally well at the two concentrations of ATP found with or without 2,4-dinitrophenol. Moreover, the percentage stimulation due to tetrodotoxin, in the presence of 1mM-sodium pyruvate, is the same with or without 2,4-dinitrophenol. These results indicate that the tetrodotoxin effect cannot be satisfactorily explained on the assumption that it acts simply by increasing the cell concentration of ATP.

Effects of time of addition of glucose on the stimulating action of tetrodotoxin on anaerobic glycolysis

When rat brain-cortex slices were incubated anaerobically in a Ca²⁺-free saline medium in the presence of 2 μM-tetrodotoxin for 15min before addition of glucose, the enhancing effect of tetrodotoxin disappeared (Table 6). The loss of response to tetrodotoxin, after a period of anaerobiosis in

the absence of glucose, does not seem to be due to permanent damage to the tissue. Thus, if the slices were briefly (e.g. 10 min) exposed to oxygen after a period of anaerobiosis (e.g. 15 min), then the ability of the slices to respond fully to tetrodotoxin, by exhibiting stimulated rates of anaerobic glycolysis, was restored.

Effects of tetrodotoxin on the concentration of Na⁺ and K⁺ in incubated cerebral-cortex slices during anoxia

The effects of tetrodotoxin on Na⁺ and K⁺ concentrations in guinea-pig brain-cortex slices incubated anaerobically in a Ca²⁺-free Locke-bicarbonate medium containing 20mM-glucose at different time-intervals from the start of anoxia are shown in Fig. 4. The presence of tetrodotoxin brought about a lowering of the tissue content of Na⁺ and an increase

in the tissue content of K^+ . Maximum effects were observed within 20 min from the start of anoxia. Similar results were obtained with rat brain-cortex slices.

The effects of tetrodotoxin on the contents of Na^+ and K^+ of the incubated brain slices were made more manifest by a modification of the experimental technique. This consisted in exposing rat brain slices, incubated at $37^\circ C$ in a Ca^{2+} -free Locke-bicarbonate medium containing 20 mM-glucose, with or without tetrodotoxin, to a preliminary period (10 min) of aerobiosis [$O_2 + CO_2$ (95:5)] followed by anaerobiosis [$N_2 + CO_2$ (95:5)]. The presence of tetrodotoxin blocked the uptake of Na^+ and efflux of K^+ that normally follow the onset of anoxia (Fig. 5).

Results similar to these were obtained with the brains of newly born guinea pigs but not with the brains of infant (2-day-old) rats, where tetrodotoxin has little or no effect on the contents of Na^+ and K^+ in the anaerobically incubated brain slices.

Tetrodotoxin had no significant effect on the contents of Na^+ and K^+ of rat kidney-medulla slices

incubated anaerobically in the Ca^{2+} -free glucose-containing Locke-bicarbonate medium.

Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in Ca^{2+} -free media containing different quantities of Na^+ and K^+

When rat brain-cortex slices were incubated anaerobically at $37^\circ C$ in Ca^{2+} -free media containing 20 mM-glucose and different quantities of Na^+ or K^+ , in the absence or presence of tetrodotoxin, the magnitude of the tetrodotoxin stimulation of glycolysis depended greatly on the cation contents of the medium. Table 7 shows that when the K^+ content of the incubation medium was raised, and the Na^+ content correspondingly decreased, the rate of anaerobic glycolysis was increased but the percentage stimulating effect of tetrodotoxin was diminished. In a

Fig. 4. Effects of tetrodotoxin on the concentrations of Na^+ and K^+ of guinea-pig brain-cortex slices after anoxia

Incubations were carried out anaerobically at $37^\circ C$ in Ca^{2+} -free Locke-bicarbonate media containing 20 mM-glucose, with or without tetrodotoxin ($2 \mu M$). Tissue concentrations of Na^+ and K^+ were measured, as described in the text, at different time-intervals from the start of anoxia. □, K^+ , tetrodotoxin absent; ●, K^+ , tetrodotoxin present; ○, Na^+ , tetrodotoxin present; ■, Na^+ , tetrodotoxin absent.

Fig. 5. Effects of tetrodotoxin on the concentrations of Na^+ and K^+ of rat brain-cortex slices after anoxia

Incubations were carried out anaerobically at $37^\circ C$ in Ca^{2+} -free Locke-bicarbonate media containing 20 mM-glucose, with or without tetrodotoxin ($2 \mu M$), after a preliminary 10 min aerobic phase in which the gas atmosphere was $O_2 + CO_2$ (95:5). Anoxia commenced when this atmosphere was displaced by $N_2 + CO_2$ (95:5). Tissue concentrations of Na^+ and K^+ were measured, as described in the text, at different time-intervals from the start of anoxia. □, K^+ , tetrodotoxin absent; ●, K^+ , tetrodotoxin present; ○, Na^+ , tetrodotoxin present; ■, Na^+ , tetrodotoxin absent.

Table 7. *Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices incubated in media containing different quantities of Na⁺ and K⁺, but free of Ca²⁺*

Rat brain-cortex slices were incubated anaerobically at 37°C in Ca²⁺-free Locke-bicarbonate media containing 20mM-glucose, in which the contents of Na⁺ and K⁺ were varied as shown below, in both the presence and the absence of tetrodotoxin (2μM). When no Na⁺ was present, NaHCO₃ was replaced by KHCO₃. Iso-osmolarity was maintained, where necessary, by addition of an appropriate quantity of sucrose. Lactate was measured manometrically over an incubation period of 1 h commencing 20 min after the start of incubation.

Cations present in the incubation medium		Lactate formed (μmol/g initial wet wt.)	
Na ⁺ (mequiv./l)	K ⁺ (mequiv./l)	Tetrodotoxin absent	Tetrodotoxin present
149	5	21 ± 1.2	72 ± 4.3
89	60	59 ± 1.2	70 ± 4.2
29	60	127 ± 7.6	144 ± 8.5
29	125	107 ± 7.1	130 ± 9.0
0	154	115 ± 5.8	114 ± 7.7

medium devoid of Na⁺, and containing 154 mequiv. of K⁺/l, tetrodotoxin had no significant effect on the rate of anaerobic glycolysis.

Effects of addition of 100 mM-KCl or 100 mM-NaCl on tetrodotoxin-stimulated glycolysis of rat brain-cortex slices

In view of the above results, experiments were carried out to observe the effects of the addition of a high (100 mM) concentration of KCl or of NaCl on the tetrodotoxin-stimulated rate of glycolysis of rat brain-cortex slices incubated anaerobically in a Ca²⁺-free Locke-bicarbonate medium. The addition of 100 mM-KCl to a medium already containing 149 mequiv. of Na⁺/l brought about a considerable depression of the rate of anaerobic glycolysis (Fig. 6). Addition of 100 mM-NaCl had only a relatively small depressing effect. Addition of sucrose (up to 200 mM) had no significant effect. Preliminary experiments indicate that the addition of 100 mM-KCl leads to an increased cell content of Na⁺, as is also known to occur under aerobic conditions (Okamoto & Quastel, 1970).

Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of EDTA or EGTA

Investigations were made of the effects of the addition to the Ca²⁺-free incubation media of substances known to bring about, under aerobic conditions, increased concentrations of Na⁺ in incubated rat brain-cortex slices. Among such substances are EDTA and EGTA, which chelate Ca²⁺ and thereby decrease to very low concentrations the small amount of ionizable Ca²⁺ still present in the 'calcium-free' Locke-bicarbonate media used in the present investigations. It is known that in the presence of

Fig. 6. *Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices after addition of 100 mM-NaCl and 100 mM-KCl*

Incubations were carried out anaerobically at 37°C in a Ca²⁺-free Locke-bicarbonate medium containing 20 mM-glucose in the presence or absence of tetrodotoxin (2μM). The arrow indicates the time at which 100 mM-NaCl or 100 mM-KCl was added to the medium. ■, Tetrodotoxin present, no other additions; ○, tetrodotoxin present, 100 mM-NaCl added; ▲, tetrodotoxin present, 100 mM-KCl added; ●, tetrodotoxin absent, no addition.

such chelating agents there is a rapid influx of Na⁺ into and rapid efflux of K⁺ from cerebral tissues, incubated aerobically in the presence of glucose, and

that the presence of tetrodotoxin has but little effect on these fluxes (Pull *et al.*, 1970).

Table 8 shows that the stimulating effect of tetrodotoxin on the anaerobic glycolysis of cortex slices from rat or guinea-pig brain incubated at 37°C in a calcium-free Locke-bicarbonate medium was greatly decreased or completely disappeared in the presence of 1 mM-EDTA or 1 mM-EGTA.

Effects of tetrodotoxin on the anaerobic glycolysis of rat brain-cortex slices in the presence of protoveratrine

Wollenberger (1955) has shown that protoveratrine inhibits anaerobic glycolysis of incubated brain slices. It brings about marked changes in ion permeability and bioelectric phenomena (Shanes, 1958), affecting the kinetics of cerebral glucose metabolism (Kini & Quastel, 1960) and inducing an influx of Na⁺ into aerobically incubated brain slices that is abolished by the presence of tetrodotoxin (Okamoto & Quastel, 1970). Protoveratrine (5 μM) decreased stimulation of anaerobic glycolysis of rat brain-cortex slices in a Ca²⁺-free medium containing 20 mM-glucose, brought about by tetrodotoxin (2 μM) (Table 9). At a very high concentration (20 μM) of protoveratrine, tetrodotoxin (2 μM) had little or no effect.

Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of sodium L-glutamate or of ammonium chloride

Sodium L-glutamate, well known to cause excitation and depolarization of the nerve cell, brings about an increased content of Na⁺ in brain-cortex

slices incubated aerobically in glucose-saline media, which is only partly diminished by the presence of tetrodotoxin (Pull *et al.*, 1970; Okamoto & Quastel, 1970).

The stimulating action of tetrodotoxin (2 μM) on anaerobic glycolysis of rat brain-cortex slices in the Ca²⁺-free medium was almost abolished by the presence of 5 mM-sodium L-glutamate (Table 10). Ammonium chloride (5 mM) also diminished very considerably the stimulation of anaerobic glycolysis due to tetrodotoxin (Table 10).

Determinations were made of the Na⁺ and K⁺ contents of rat brain-cortex slices incubated anaerobically, with or without tetrodotoxin in the Ca²⁺-free medium, after a preliminary 10 min aerobic phase, in both the presence and the absence of either sodium L-glutamate (5 mM) or of ammonium chloride (5 mM). Results given in Table 11 show that the retention of K⁺ and diminished influx of Na⁺, characteristic of the action of tetrodotoxin, under the given experimental conditions took place much less markedly in the presence of L-glutamate or of ammonium chloride. The effects of L-glutamate or of ammonium chloride in blocking the enhancing effect of tetrodotoxin on cerebral anaerobic glycolysis would seem to be related to their effects on the fluxes of Na⁺ and K⁺ in the incubated brain slices.

Effects of tetrodotoxin on the anaerobic uptakes of L-[U-¹⁴C]glutamate and of [2-¹⁴C]glycine by rat brain-cortex slices under anoxia

Since tetrodotoxin brings about a diminution of the Na⁺ content and an increase of the K⁺ content of

Table 8. *Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of EDTA or EGTA*

Cortex slices from rat or guinea-pig brain were incubated anaerobically at 37°C in a Ca²⁺-free Locke-bicarbonate medium containing 20 mM-glucose in the presence or absence of EDTA (1 mM) or EGTA (1 mM) or of tetrodotoxin (2 μM). Lactate formation was measured manometrically over a period of 1 h commencing 20 min after the start of incubation.

Additions to the incubation medium	Lactate formed (μmol/g initial wet wt.)	
	Rat	Guinea pig
Nil	24.4 ± 2.8	30.7 ± 7.6
Tetrodotoxin	81.1 ± 7.6	141.8 ± 14.5
EDTA	11.3 ± 1.0	11.8 ± 0.8
EDTA + tetrodotoxin	17.8 ± 4.3	14.9 ± 4.6
EGTA	24.9 ± 3.5	17.7 ± 2.9
EGTA + tetrodotoxin	27.3 ± 1.9	37.5 ± 8.0

Table 9. *Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of protoveratrine*

Rat brain-cortex slices were incubated anaerobically at 37°C in a Ca²⁺-free Locke-bicarbonate medium containing 20 mM-glucose in the presence of tetrodotoxin or protoveratrine or mixtures of these substances. Lactate formation was measured manometrically over a period of 1 h commencing 20 min after the start of the incubation.

Additions to the incubation medium (μM)	Lactate formed (μmol/g initial wet wt.)
Nil	17.9 ± 3.6
Tetrodotoxin (2)	55.4 ± 7.3
Protoveratrine (5)	10.3 ± 0.9
Tetrodotoxin (2) + protoveratrine (5)	31.7 ± 4.9
Tetrodotoxin (10) + protoveratrine (5)	43.8 ± 3.6

rat brain-cortex slices incubated anaerobically in a Ca^{2+} -free glucose-containing medium, investigations were carried out to observe whether tetrodotoxin exerts similar effects on amino acid uptake and efflux under such anaerobic conditions. No inhibition by tetrodotoxin of amino acid (L-glutamate, glycine) uptake was found, but, on the contrary, tetrodotoxin appeared to accelerate it (Table 12). Although the acceleration was not large, and not comparable in magnitude with the acceleration of the rate of anaerobic glycolysis, it was nevertheless significant.

Table 10. *Effects of tetrodotoxin on anaerobic glycolysis of rat brain-cortex slices in the presence of sodium L-glutamate and of ammonium chloride*

Rat brain-cortex slices were incubated anaerobically at 37°C in a Ca^{2+} -free Locke-bicarbonate medium containing 20mm-glucose, in the presence or absence of sodium L-glutamate (5mm), ammonium chloride (5mm) or tetrodotoxin ($2\mu\text{M}$). Lactate formation was measured manometrically over a period of 1h commencing 20min after the start of incubation.

Additions to the incubation medium	Lactate formed ($\mu\text{mol/g}$ initial wet wt.)	
	Tetrodotoxin absent	Tetrodotoxin present
Nil	28.3 ± 4.3	67.9 ± 6.7
Sodium L-glutamate	20.9 ± 2.3	22.4 ± 2.5
Ammonium chloride	29.9 ± 5.8	37.0 ± 4.3

The reason appears to be that tetrodotoxin has a marked suppressing effect on the efflux of amino acids from rat brain-cortex slices incubated anaerobically in a Ca^{2+} -free glucose medium. This was shown as follows.

Effects of tetrodotoxin on the amino acid contents of rat brain-cortex slices incubated under anoxia

When rat brain-cortex slices were incubated at 37°C anaerobically in the Ca^{2+} -free medium containing 20mm-glucose there was a relatively rapid efflux of glutamate, aspartate and glycine from the slices into the medium. If the incubation was carried out in the presence of $2\mu\text{M}$ -tetrodotoxin the rate of disappearance of these amino acids from the tissue into the medium was retarded (Table 13). This was particularly so with glutamate.

It appears therefore that tetrodotoxin is not only effective in causing retention of K^+ in rat brain slices incubated anaerobically in a Ca^{2+} -free medium, but it also causes retention in the tissue of certain amino acids, particularly glutamate and aspartate.

Effects of local anaesthetics on anaerobic glycolysis of rat brain-cortex slices

The effect of local anaesthetics on cerebral metabolism is known to resemble that of tetrodotoxin in certain respects; for example, they block the increased influx of Na^+ and decrease of acetate oxidation caused by electrical stimulation (Chan & Quastel, 1970) and appear to decrease Na^+ conductance (Goldman &

Table 11. *Effects of tetrodotoxin on the contents of Na^+ and K^+ of rat brain-cortex slices incubated anaerobically in the presence of sodium L-glutamate (5mm) or of ammonium chloride (5mm)*

Rat brain-cortex slices were incubated at 37°C in a Ca^{2+} -free Locke-bicarbonate medium containing 20mm-glucose, with or without tetrodotoxin ($2\mu\text{M}$), after a preliminary 10min aerobic phase in which the gas atmosphere was $\text{O}_2 + \text{CO}_2$ (95:5). Anoxia commenced when this atmosphere was displaced by $\text{N}_2 + \text{CO}_2$ (95:5). Tissue concentrations of Na^+ and K^+ were measured as described in the text at different time-intervals from the start of anoxia.

Additions to the incubation medium	Time from start of anoxia (min)	Concn. of ion ($\mu\text{equiv./g}$ initial wet wt. of tissue)			
		Tetrodotoxin absent		Tetrodotoxin present	
		Na^+	K^+	Na^+	K^+
Nil	5	183 ± 22	29 ± 1	141 ± 13	54 ± 3
	10	209 ± 36	20 ± 2	140 ± 13	46 ± 2
	30	222 ± 8	15 ± 1	140 ± 5	44 ± 3
Sodium L-glutamate	5	170 ± 9	25 ± 1	155 ± 5	42 ± 1
	10	195 ± 8	21 ± 1	175 ± 5	37 ± 1
	30	220 ± 10	16 ± 1	220 ± 10	23 ± 1
Ammonium chloride	5	145 ± 5	24 ± 1	125 ± 7	35 ± 1
	10	170 ± 15	16 ± 1	140 ± 5	30 ± 1
	30	210 ± 7	15 ± 1	185 ± 9	18 ± 3

Table 12. *Effects of tetrodotoxin on the anaerobic uptakes of sodium L-[U-¹⁴C]glutamate and of [2-¹⁴C]glycine by rat brain-cortex slices under anoxia*

Rat brain-cortex slices were incubated anaerobically at 37°C in a Ca²⁺-free Locke-bicarbonate medium containing 20 mM-glucose in the presence or absence of tetrodotoxin (2 μM), for a preliminary period of 15 min, and then in the presence of 5 mM-sodium L-[U-¹⁴C]glutamate or of 2 mM-[2-¹⁴C]glycine for periods of 15 min or 30 min. After termination of these periods, the uptakes of radioactivity were measured as described in the text. The uptakes are expressed in terms of μmol of glutamate or glycine/g calculated by dividing the c.p.m./g present in the tissue by the specific radioactivity of the amino acid in the medium.

Additions to the incubation medium	Uptake of amino acid (μmol/g initial wet wt.)			
	Tetrodotoxin absent		Tetrodotoxin present	
	15 min	30 min	15 min	30 min
Sodium L-[U- ¹⁴ C]glutamate	4.18 ± 0.25	5.16 ± 0.30	5.56 ± 0.28	6.85 ± 0.35
[2- ¹⁴ C]Glycine	1.85 ± 0.10		2.86 ± 0.16	

Blaustein, 1966). Experiments were therefore carried out to determine whether they affect the rate of anaerobic glycolysis of brain slices in a manner similar to that of tetrodotoxin. The results showed that both procaine (0.01 mM) and lidocaine (0.01 mM) bring about a marked acceleration (162% with procaine and 184% with lidocaine) of anaerobic glycolysis under conditions where tetrodotoxin (2 μM) brings about an acceleration of 300%. Further experiments showed that the local anaesthetics resemble tetrodotoxin in being effective only when present at the onset of anoxia and in their causing retention of K⁺ in the tissue when the rat brain slices are incubated in a Ca²⁺-free glucose-containing medium.

The results of experiments carried out with the brains of 2-day-old rats and newly born guinea pigs are given in Fig. 7. They show that lidocaine has a marked stimulating effect on the anaerobic glycolysis of the infant guinea-pig brain but not that of infant rat brain. This behaviour again resembles that due to tetrodotoxin.

Effects of sodium Amytal on the anaerobic glycolysis of rat brain-cortex slices

Experiments were carried out to observe whether a barbiturate, sodium Amytal, affects the rate of anaerobic glycolysis of rat brain-cortex slices incubated anaerobically in either Ca²⁺-free or Ca²⁺-containing media. Amytal, in contrast with tetrodotoxin, exerted little enhancing effect on the rate of anaerobic glycolysis of brain slices until its concentration reached the relatively high value of 1 mM (Table 14). In Ca²⁺-free media, Amytal was more effective than in Ca²⁺-containing media, but even under such conditions Amytal, at the concentration of 0.25 mM, which is the concentration found in the blood of rats under Amytal anaesthesia (Reading &

Wallwork, 1969), gave only a relatively small increase in glycolytic rate compared with that caused by tetrodotoxin or the local anaesthetics at the concentrations quoted above. It was evident, however, that the stimulating action of Amytal on cerebral anaerobic glycolysis was greatly dependent on the Ca²⁺ concentration of the medium.

Effect of pyrrole on the anaerobic glycolysis of rat brain-cortex slices

In view of the findings of Adams & Quastel (1956) that pyrrole increases the rate of anaerobic glycolysis of guinea-pig brain-cortex slices incubated in a Ca²⁺-free glucose-containing medium, experiments were carried out to observe whether pyrrole had effects resembling tetrodotoxin. It was found that pyrrole (30 mM) has properties very much in common with tetrodotoxin (2 μM) so far as metabolism of brain slices under anoxia is concerned. It accelerated anaerobic glycolysis of guinea-pig brain-cortex slices incubated in a Ca²⁺-free media by 95%, but only when it was added at the onset of anoxia. Results given in Fig. 8 show that pyrrole (30 mM) is capable of decreasing the Na⁺ content and of increasing the K⁺ content of rat brain-cortex slices in a manner resembling that of tetrodotoxin.

Discussion

It was found by Adams & Quastel (1956), after earlier work by Quastel & Wheatley (1937), that the anaerobic glycolysis of brain-cortex slices is markedly stimulated by the addition of calcium salts to a medium containing Na⁺ and K⁺. It is known that Ca²⁺ is necessary for the maintenance of the normal permeability of cell plasma membranes (Shanes, 1958; Whitembury *et al.*, 1960; Curran

Table 13. *Effects of tetrodotoxin on the amino acid content of rat brain-cortex slices incubated under anoxia*

Rat brain-cortex slices were incubated anaerobically at 37°C for 30min in a Ca²⁺-free Locke-bicarbonate medium containing 20mM-glucose in the presence or absence of tetrodotoxin (2 μ M). The tissues and the incubation medium were then analysed for their contents of glutamate, aspartate and glycine by methods described in the text.

Addition to the incubation medium	Amino acids present (μ mol/g initial wet wt.)					
	Glutamate		Aspartate		Glycine	
	Tissue	Medium	Tissue	Medium	Tissue	Medium
Nil	6.67 $\pm$ 0.50	6.10 $\pm$ 0.34	0.96 $\pm$ 0.05	1.24 $\pm$ 0.2	0.41 $\pm$ 0.05	0.62 $\pm$ 0.04
Tetrodotoxin	9.10 $\pm$ 0.20	3.54 $\pm$ 0.44	1.65 $\pm$ 0.02	0.82 $\pm$ 0.17	0.57 $\pm$ 0.02	0.46 $\pm$ 0.04

Fig. 7. *Effects of lidocaine on anaerobic glycolysis of slices from infant rat or infant guinea-pig brain cortex*

Incubations were carried out anaerobically at 37°C in a Ca²⁺-free Locke-bicarbonate medium. Lactate was measured manometrically over a period of 1h commencing 20min after the start of the experiment. Lidocaine at various concentrations was present from the beginning of the experiment. ○, Rat (2 days old); ●, guinea pig (newly born).

et al., 1963) and that if animal cells are incubated in media free of Ca²⁺ they are unable to maintain their normal concentration gradients of Na⁺ and K⁺ (Judah & Ahmed, 1963; Kleinzeller *et al.*, 1968). Deprivation of Ca²⁺ from the incubation medium

causes liver slices (Van Rossum, 1970) and brain slices (Joanny & Hillman, 1964) to lose K⁺ and gain Na⁺. We have also found that there is increased tissue retention of K⁺ on addition of Ca²⁺ to rat brain-cortex slices incubated anaerobically in a Ca²⁺-free glucose-saline medium; for example, a value of 15 μ equiv. of K⁺/g, found after 30min anaerobic incubation at 37°C of rat brain-cortex slices incubated in a Ca²⁺-free Locke-bicarbonate medium containing 20mM-glucose, was increased to 25 μ equiv. of K⁺/g by the addition of 4mM-calcium chloride to the incubation medium.

The present results show that tetrodotoxin, at concentrations that block the generation of action potentials and the associated influx of Na⁺ (Kao, 1966), brings about a marked rise (which may exceed 300%) in the rate of anaerobic glycolysis of incubated slices from rat or guinea-pig brain. Moreover, the effect of tetrodotoxin occurs with adult rat or guinea-pig brain slices and with newly born guinea-pig brain slices but not with infant rat brain slices. It is well known that infant guinea-pig brain shows mature characteristics (Dobbing, 1970), and it is therefore evident that the action of tetrodotoxin is closely associated with the maturity of the brain tissue. Tetrodotoxin does not affect the glycolytic process itself.

The effect of tetrodotoxin occurs in a Ca²⁺-containing incubation medium, as well as in a Ca²⁺-deficient medium, but its activity is diminished in the presence of Ca²⁺. The results show that tetrodotoxin does not simply replace Ca²⁺ in bringing about an acceleration of glycolysis, but that the actions of the two substances are similar and may be directed towards the same rate-limiting reaction in the glycolytic process.

The effect of tetrodotoxin seems not to be dependent on the cell concentration of pyruvate or of ATP. Nevertheless, when the cell concentration of ATP falls so low that the phosphorylation of glucose becomes rate-limiting, tetrodotoxin has no effect. Presumably this occurs when anoxia is allowed to take

Table 14. *Effects of Amytal on the anaerobic glycolysis of rat brain-cortex slices incubated in Ca^{2+} -free and Ca^{2+} -containing media*

Rat brain-cortex slices were incubated anaerobically at 37°C either in Ca^{2+} -free Locke-bicarbonate media or in Krebs-Ringer bicarbonate media, containing 20mM-glucose in the presence of various concentrations of sodium Amytal. Lactate formation was measured manometrically over a period of 1 h commencing 20 min after the start of the incubation.

Additions to the incubation medium (mM-Amytal)	Lactate formed ($\mu\text{mol/g}$ initial wet wt.)	
	Ca^{2+} -free medium	Ca^{2+} -containing medium
Nil	30.0 ± 2.5	38.4 ± 3.6
0.25	47.5 ± 3.0	34.8 ± 7.6
0.5	58.5 ± 3.6	39.7 ± 1.8
1.0	80.0 ± 4.1	54.5 ± 2.3

place for 15 min (in the presence of tetrodotoxin) before glucose is added (Table 6). We find that, under such circumstances, the ATP concentration falls to 0.35mM. As the K_m of ATP for brain-cortex hexokinase is 0.56mM (Thompson & Bachelard, 1970), it is evident that at the low ATP concentration of 0.35mM the hexokinase reaction may be rate-limiting for glycolysis.

The major effect of tetrodotoxin, at the onset of anoxia, on processes taking place in brain slices incubated in a glucose-saline medium seems to consist of a suppression of influx of Na^+ and of efflux of K^+ (Fig. 5). That this effect is closely related to the stimulating action of tetrodotoxin on the rate of glycolysis, at the onset of anoxia, is borne out by the fact that the tetrodotoxin effect disappears when the brain slices are incubated anaerobically in a medium in which Na^+ has been replaced by K^+ (Table 7). Such a relation also makes it possible to understand why the enhancing effect of tetrodotoxin is greatly decreased in an incubation medium containing EDTA or EGTA (Table 8) where the influx of Na^+ and efflux of K^+ are large and almost insensitive to tetrodotoxin, or in the presence of protoveratrine (Table 9), which promotes the influx of Na^+ (Okamoto & Quastel, 1970), or in the presence of sodium L-glutamate or of ammonium chloride (Tables 10 and 11), both of which promote an influx of Na^+ and an efflux of K^+ , whose contents in the brain are but little affected by tetrodotoxin.

These facts make it reasonable to conclude that the tetrodotoxin effect on brain glycolysis, at the onset of anoxia, is directly due to the action of tetrodotoxin in suppressing the efflux of K^+ and the influx of Na^+ that occur as soon as anoxia commences. Such a conclusion also makes it possible to understand why the

Fig. 8. *Effects of pyrrole on the concentrations of Na^+ and K^+ of incubated guinea-pig brain-cortex slices under anoxia*

Incubations were carried out anaerobically at 37°C in a Ca^{2+} -free Locke-bicarbonate medium containing 20mM-glucose in the presence or absence of pyrrole (30mM). Na^+ and K^+ were measured as described in the text at various times after the start of the experiment. ■, K^+ , pyrrole absent; □, K^+ , pyrrole present; ○, Na^+ , pyrrole present; ●, Na^+ , pyrrole absent.

addition of 100mM-KCl to a medium in which anaerobic glycolysis, in the presence of tetrodotoxin, is proceeding brings about a suppression of the rate of glycolysis (Fig. 6). The inhibitory effect of the increased cell concentration of Na^+ , due to the increased K^+ in the medium, may then outweigh the stimulatory effect of K^+ .

The increased rate of anaerobic glycolysis by rat brain slices caused by an increased cell concentration of K^+ and decreased cell concentration of Na^+ may perhaps be mediated by the activity of pyruvate kinase, which is well known to be affected by K^+ (Lardy & Ziegler, 1945). The activity of this enzyme is doubled by doubling the K^+ concentration within a range of 0–50mequiv. of K^+ /l (Takagaki, 1968) and the activation by K^+ is diminished by Na^+ (Rose & Rose, 1969). But whether this is the only enzyme operating in the glycolytic pathway, affected by K^+ , which will account for the accelerative action of tetrodotoxin on cerebral anaerobic glycolysis is a matter for further investigation.

As mentioned above, tetrodotoxin suppresses the influx of Na^+ in incubated rat brain-cortex slices under a variety of incubation conditions (Okamoto & Quastel, 1970) and it was concluded that, under such conditions, generation of action potentials must occur with concomitant influx of Na^+ and efflux of K^+ . To these conditions must now be added that occurring in brain at the onset of anoxia. It seems clear that the fluxes of Na^+ and K^+ that accompany the generation of action potentials taking place at the onset of anoxia are suppressed by the presence of tetrodotoxin.

It has been pointed out (Okamoto & Quastel, 1970) that tetrodotoxin does not affect the fluxes of Na^+ and K^+ taking place when brain slices are incubated aerobically in a Krebs-Ringer-glucose medium and that, under such circumstances, action potentials are not generated. This fact makes it possible to understand why tetrodotoxin has no stimulating effect on the rate of aerobic glycolysis of rat brain slices incubated in a physiological saline-glucose medium.

Tetrodotoxin is not only effective in causing retention of K^+ in brain slices incubated anaerobically in a Ca^{2+} -free Locke-bicarbonate medium, but it also causes diminished efflux of certain amino acids, particularly glutamate and aspartate (Tables 12 and 13). This result is in accord with the conclusion, derived from other experiments in this laboratory (Benjamin & Quastel, 1971), that the generation of action potentials in incubated rat brain-cortex slices is accompanied by an increased efflux of amino acids, particularly glutamate and aspartate, from the tissue into the incubation medium.

The local anaesthetics (procaine, lidocaine), at low concentrations, also bring about effects on the anaerobic glycolysis of rat brain-cortex slices that closely resemble those of tetrodotoxin. These results, together with those found with tetrodotoxin on the metabolism and Na^+ fluxes of electrically stimulated brain slices (Chan & Quastel, 1970), support the conclusion that the local anaesthetics suppress the generation of action potentials in isolated brain tissue and the concomitant metabolic changes.

Amytal, at the anaesthetic concentration (for the rat) of 0.25 mM, seems not to behave in this manner. It has but little effect on the anaerobic glycolysis of rat brain-cortex slices under conditions where tetrodotoxin and local anaesthetics have marked effects and at the relatively low concentration that blocks electrical and K^+ stimulation of brain respiration (Chan & Quastel, 1970). However, Amytal exerts a significant effect in stimulating anaerobic glycolysis of rat brain-cortex slices in a Ca^{2+} -free medium. It is suggested that this effect of Amytal is brought about, at the onset of anoxia, by its well-known suppressing action on mitochondrial metabolism, liberating Ca^{2+} from its bound state in the mitochondria and increasing the cell concentration of ionizable Ca^{2+} (Chappell

& Crofts, 1965). Presumably this will result in changed membrane fluxes of Na^+ and K^+ that may resemble those taking place when the brain slices are incubated in a Ca^{2+} -containing medium.

Stimulation of the rate of glycolysis of rat brain-cortex slices incubated anaerobically in a Ca^{2+} -free saline medium is not specific for anaesthetics. Pyrrole (30 mM), already known to accelerate anaerobic glycolysis of guinea-pig brain-cortex slices, has effects resembling those of tetrodotoxin, though it is only effective at very much higher concentrations.

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