3. R. W. Butcher and E. W. Sutherland, *ibid.*, p. 1244.
4. L. M. Klainer, *et al.*, *ibid.*, p. 1239.
5. U. Trendelenburg, *Pharmacol. Rev.* **18**, 629 (1966).
6. J. Ariens Kappers, *Z. Zeilforsch. Mikroskop. Anat.* **52**, 163 (1960).
7. R. Hakanson and C. Owman, *J. Neurochem.* **12**, 417 (1965).
8. C. Owman, *Int. J. Neuropharmacol.* **2**, 105 (1964).
9. L. M. Zieher and A. Pellegrino de Iraldi, *Acta Physiol. Latinoamer.* **16**, 298 (1966).
10. G. Krishna, B. Weiss, B. B. Brodie, in preparation.
11. B. Weiss and E. Costa, *Fed. Proc.* **26**, 765 (1967).
12. B. Weiss, J. I. Davies, B. B. Brodie, *Biochem. Pharmacol.* **15**, 1553 (1966).
13. G. Rodriguez de Lores Arnaiz, *Acta Physiol. Latinoamer.* **16**, 303 (1966).
14. R. J. Wurtman, J. Axelrod, J. E. Fischer, *Science* **143**, 1328 (1964); E. G. McGeer and P. L. McGeer, *ibid.* **153**, 73 (1966).
15. Supported by PHS grants NB 05184-03 and PH-436454. We thank R. Lehne for technical assistance.

22 March 1967

Tetrodotoxin: Effects on Brain Metabolism in vitro

Abstract. *A 3-micromolar concentration of tetrodotoxin completely inhibits the stimulation of respiration of rat brain cortex that takes place upon application of electrical impulses. It also inhibits increase in the rate of the respiration that occurs when calcium ions are omitted from the incubation medium. No effect of tetrodotoxin on brain respiration takes place when stimulation is brought about by the addition of 100 millimolar potassium chloride. Tetrodotoxin prevents the fall in the rate of oxidation of cerebral acetate that occurs during electrical stimulation but does not affect the increased rate of the oxidation that occurs in the presence of an increased concentration of potassium chloride. The data indicate that oxidation of cerebral acetate is diminished by influx of sodium ions, which is prevented by tetrodotoxin, and is increased by influx of potassium ions, which is unaffected by tetrodotoxin.*

Tetrodotoxin (TTX), a potent neurotoxin found in the Japanese puffer fish and elsewhere (*1*), in low concentrations, such as 0.3 μM, suppresses action potentials in a variety of excitable tissues, including frog myelinated nerve fibers and lobster and squid giant axons (*2–4*). It is considered to be a specific blocker of sodium conductance, but with no effect on potassium conductance (*3–6*). It does, however, have suppressing effects on action potentials produced in media containing salts of divalent cations only (Ca^{++}, Sr^{++}, and Ba^{++}) (*7, 8*).

We have carried out experiments to observe whether tetrodotoxin affects the metabolism and transport reactions of brain cortex slices incubated aerobically in a physiological Ringer medium. It is well known that various aspects of the metabolism of isolated brain tissue are greatly influenced by changes in the cation concentration of the incubation medium or by application of electrical impulses (*9*). It seemed possible that use of tetrodotoxin might help to throw further light on the mechanisms whereby cations secure, in part, control of brain cell metabolism.

Metabolism and transport reactions in slices of brain cortex from adult hooded rats were investigated by methods already described (see *10*). Cortex slices (70 to 80 mg) were cut from the cerebral hemispheres with a Stadie-Riggs tissue slicer, care being taken to make the slices not more than 0.3 mm thick, but not so thin that they would tend to disintegrate when shaken in the Warburg manometric apparatus in which incubations were usually conducted. All incubations were normally carried out at 37°C for 1 hour in an atmosphere of oxygen in a Krebs-Ringer medium containing 128 mM NaCl, 5.0 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 10 mM Na_2HPO_4 (brought to *p*H 7.4 with 1.0N HCl), and 10 mM glucose, the final volume in all cases being 3 ml. When potassium ions were increased, potassium chloride was added to the normal medium to the desired concentration. For a calcium-free medium, calcium chloride was omitted from the normal medium. In some experiments, 2 mM sodium acetate-1-^{14}C was added to the normal medium at the commencement of the experiment and the amount of $^{14}CO_2$ evolved at the end of the incubation period was estimated by methods previously described (*11*).

Electrical stimulation of slices of brain cortex was carried out in vessels with silver grid electrodes similar to those used by Wallgren and Kulonen (*12*), with electrical impulses from the alternating frequency generator of the Teratron type, described in detail by Wallgren (*13*). The impulses had a pulse frequency of 100 cycle/sec with 1-msec duration, the peak potential being 4 volts. The impulses were displayed on a type 561A oscilloscope during the experiments.

Studies were made of the velocities of oxygen consumption in the presence or absence of TTX (Sankyo Co.) used at a concentration of 3 μM. Measurements were also made of the rates of evolution of $^{14}CO_2$ in the presence or absence of TTX in media containing 2 mM sodium acetate-1-^{14}C. The results are given in Table 1 which records, for each condition, the means of four independent observations ± standard deviation.

These results show that while TTX has no effect, within experimental error, on the rate of respiration of slices of adult rat brain cortex incubated in a physiological Ringer medium containing glucose, it completely blocks the stimulation of respiration that takes place on application of electrical impulses. An equal inhibitory effect of TTX occurs at lower concentrations (for example, 0.3 μM). Table 1 shows that TTX inhibits, almost completely, the increase in the rate of rat brain cortex respiration that occurs when calcium ions are omitted from the medium. Thus, TTX may exert an effect on brain metabolism in vitro independently of the application of electrical impulses. When the rate of brain respiration is increased by the addition of 100 mM KCl, no effect of TTX within experimental error is observed. On the basis of results given in Table 1, we conclude that, in the presence of 3 μM TTX, neither electrical stimulation nor the absence of calcium ions will cause enhancement of brain cell respiration, but that the addition of high concentrations of potassium ions is equally as effective, in stimulating brain respiration, in the presence as in the absence of TTX.

These results may be interpreted as indicating that, with electrical stimulation, there is an increased influx into the brain cell of sodium ions (*14*) which, by their accelerating effect on the activity of membrane adenosine triphosphatase, result in an increased rate of brain cell respiration controlled by adenosine diphosphate. The TTX suppresses, because of its combination with a specific membrane component, this response of the brain cell to an applied electrical impulse. Presumably, in the absence of calcium ions from the incubating medium, there is also an increased influx of sodium ions (*15*) mediated by the TTX-sensitive component of the cell membrane. It is known that removal of divalent cations from the external medium may lead to marked fluctuations of the membrane potential and to spontaneous excitation (*8*).

In accordance with the observation that TTX does not affect potassium conductance, there is no effect within experimental error of TTX on potas-

Table 1. Effects of tetrodotoxin on the metabolism of slices of rat brain cortex. All tissue was incubated for 1 hour at 37°C in oxygen in a medium consisting of Krebs-Ringer solution that contained phosphate and glucose.

Changes in conditions	No drug added	TTX added (3 μM)
Oxygen consumed (μl per mg dry wt. of tissue per hour)		
None	12.0 ± 0.3	12.5 ± 0.6
Electrical stimulation	15.6 ± .3	12.3 ± .3
Ca^{++} omitted	15.4 ± .1	13.0 ± .3
KCl (100 mM) added	17.0 ± .8	16.6 ± .8
$^{14}CO_2$ evolved (mμg mole per 100 mg wet wt. of tissue per hour)		
Acetate-1-^{14}C added	88 ± 6	82 ± 6
Acetate-1-^{14}C added and with electrical stimulation	51 ± 3	80 ± 4
Acetate-1-^{14}C and KCl (100 mM) added	141 ± 5	139 ± 6

sium-stimulated brain respiration. In this case, the flux of potassium ions, which activate the membrane adenosine triphosphatase and thereby stimulate brain cell respiration, is unimpeded by TTX.

Results obtained by the use of labeled acetate are of importance in connection with these conclusions. The rate of evolution of $^{14}CO_2$ from acetate-1-^{14}C in the presence of slices of rat brain cortex in a Krebs-Ringer medium containing glucose is increased by the addition of potassium ions, and it is a sodium-dependent, ouabain-sensitive process (*10*). In addition, the conversion of acetate to acetyl coenzyme A, a necessary preliminary to acetate oxidation, is stimulated by potassium ions and inhibited by sodium ions (*16*). It follows that the rate of acetate oxidation by the brain cell will be dependent on the operation of two factors: (i) influx of potassium ions or efflux of sodium ions, either of which will increase the rate of conversion of acetate to acetyl coenzyme A, and (ii) the activity of membrane adenosine triphosphatase that will partly control the rate of respiration and that is both sodium- and potassium-dependent. It would be expected that TTX would not affect potassium stimulation of acetate oxidation, as it does not impede the flux of potassium ions. Results given in Table 1 confirm this. However, it might be expected that electrical stimulation, by increasing influx of sodium ions, would suppress acetate oxidation. That such suppression occurs is already known (*17*). The TTX, by its suppression of sodium conductance, should therefore block the inhibitory effect of electrical stimulation on acetate oxidation to CO_2. Results of experiments to test this conclusion are given in Table 1 and show that TTX prevents the fall in the rate of acetate oxidation that occurs upon electrical stimulation.

A variety of experiments, which will be described in detail elsewhere, have shown that TTX has no inhibitory effect on the active transport of amino acids or of phosphate ions into the brain cell. Its mode of action on metabolism, therefore, is very different from that of ouabain, which is also known to suppress electrical stimulation of brain respiration (*18*) but which, in contrast to TTX, antagonizes potassium-stimulated brain respiration (*19*).

Our results show that changes in the cation concentration of the incubation medium, or the application of electrical impulses, bring about concomitant changes in the influx or efflux of sodium and potassium ions into the brain cell with subsequent changes of brain metabolism. Therefore, TTX may be used, with advantage, for the study of those effects on brain metabolism brought about by cationic fluxes at the brain cell membrane.

S. L. CHAN
J. H. QUASTEL
Neurochemistry Section, Kinsmen Laboratories, University of British Columbia, Vancouver 8, Canada

References and Notes

1. C. Y. Kao, *Pharmacol. Rev.* **18**, 997 (1966).
2. T. Furukawa, T. Sasaoka, Y. Hosoya, *Jap. J. Physiol.* **9**, 143 (1959).
3. T. Narahashi, J. W. Moore, W. R. Scott, *J. Gen. Physiol.* **47**, 965 (1964).
4. Y. Nakamura, S. Nakajima, H. Grundfest, *ibid.* **48**, 985 (1965); *Science* **146**, 266 (1964); *Biol. Bull.* **127**, 382 (1964).
5. W. R. Loewenstein, C. A. Terzulo, Y. Washizu, *Science* **142**, 1180 (1963).
6. D. E. Goldman and M. P. Blaustein, *Ann. N.Y. Acad. Sci.* **137**, 967 (1966); H. Grundfest, *ibid.*, p. 901.
7. A. Watanabe, I. Tasaki, I. Singer, L. Lerman, *Science* **155**, 95 (1967).
8. I. Tasaki and I. Singer, *Ann. N.Y. Acad. Sci.* **137**, 792 (1966).
9. H. McIlwain, in *Neurochemistry*, K. A. C. Elliott, I. H. Page, J. H. Quastel, Eds. (Thomas, Springfield, Ill., ed. 2, 1962), p. 212; J. H. Quastel, in *ibid.*, p. 226; M. Brossard and J. H. Quastel, *Can. J. Biochem. Physiol.* **41**, 1243 (1963).
10. O. Gonda and J. H. Quastel, *Biochem. J.* **100**, 83 (1966).
11. ———, *Nature* **193**, 138 (1962).
12. H. Wallgren and E. Kulonen, *Biochem. J.* **75**, 150 (1960).
13. Teratron Co., Helsingfors, Finland; H. Wallgren, *J. Neurochem.* **10**, 349 (1963).
14. J. C. Keesey, H. Wallgren, H. McIlwain, *Biochem. J.* **95**, 289 (1965).
15. B. Frankenhauser and A. L. Hodgkin, *J. Physiol.* **128**, 40P (1955).
16. R. W. Von Korff, *J. Biol. Chem.* **203**, 265 (1953).
17. R. Lindbohm and H. Wallgren, *J. Neurochem.* **13**, 573 (1966).
18. H. Wallgren, *Ann. Med. Exp. Fenn.* **41**, Fasc. 2 (1963).
19. O. Gonda and J. H. Quastel, *Biochem. J.* **84**, 394 (1962).
20. Supported by a grant from the Medical Research Council of Canada.

22 March 1967

Regulation of the Adrenal Cortex Secretory Pattern by Adrenocorticotropin

Abstract. *Adrenal gland homogenates from rabbits stimulated for 28 days with adrenocorticotropin have been shown to synthesize cortisol. The total amount of cortisol formed was the same whether progesterone-4-^{14}C or Δ^5-pregnenolone-4-^{14}C was used as exogenous substrate. However, when Δ^5-pregnenolone substrate was used the specific activity of the isolated cortisol was four times as great as when progesterone was used.*

In 1954 Kass *et al.* (*1*) reported that the adrenal secretory pattern of the rabbit could be altered from primarily corticosterone (B_k) to primarily cortisol (F_k). This change was effected by daily stimulation with 12 to 25 units of porcine adrenocorticotropin (ACTH) for 25- to 28-day periods. Confirmation of the increased F_k secretion after similar stimulation with ACTH has been published by Yudaev and Afinogenova and by Krum and Glenn (*2*). Neither reported that there was more F_k than B_k secreted as originally found by Kass and co-workers (*1*), although the B_k secretion rate decreased in both cases. Yudaev and Morozova (*3*) later reported that results of incubations of adrenal slices from rabbits injected daily with ACTH for 24 days prior to being killed indicated that the increase in F_k with concomitant decrease in B_k secretion rates was due to a partial inhibition of the 21-hydroxylase. This partial inhibition would, according to the authors, make more progesterone available for 17α-hydroxylation. The activity of the 21-hydroxylase enzyme per gram of adrenal tissue was reported to be twice as great in tissue from control animals as in ACTH-stimulated animals. However, since the adrenal weights of experimental animals were approximately twice those of control animals, the apparent "inhibition" of the 21-hydroxylase reported to be effected by ACTH stimulation was actually only a lack of stimulation of this enzyme activity. In the present report results of studies conducted to investigate the mechanism by which ACTH stimulates F_k production by adrenal cortical tissue of the rabbit are presented. The results indicate a possible change in the metabolic pathway of pregnenolone in adrenal tissue from stimulated rabbits.