tion movements even when treated with fumigants, is described.

Nicotine when introduced in Ringer’s solution flooding the insect’s body showed complete inhibition of spiracular movements of intact, decerebrated and decapitated insects (Figure A, B and C). The second thoracic spiracle remained open with nicotine solution, indicating that nicotine brings about inhibition in the pacemakers situated in the body outside the cephalic region. But when nicotine solution was released on the brain, increased respiratory movements of the spiracles (103 beats/min) were observed (Figure D), just as in the case of the decapitated insect. Obviously, nicotine diminishes the inhibitory action of brain.

EDCT in a similar test showed increased spiracular movements with intact (30 beats/min), decerebrated (40 beats/min), and decapitated (80 beats/min) insects (Figure E, F and G), thus exhibiting its excitatory effect on pacemakers. But when released on the brain it caused reduced spiracular activity (3 beats/min) (Figure H). The reactions of EDCT are diametrically opposite to that produced by nicotine.

Decerebration increased spiracular activity (23 beats per minute) and decapitation resulted in further increase (104 beats/min) (Figure J and K). This indicates that brain inhibits spiracular movements. Both cerebral and suboesophageal ganglia are inhibitory in action. This was further confirmed by releasing brain extract (5 brains homogenized in 0.5 ml and diluted in 200 ml of Ringer’s solution) on the decapitated insect. The increased spiracular activity resulting from decapitation was inhibited to almost normal (Figure I and L), indicating that an unidentified neurohormone is involved in the spiracular regulation of respiration.

Zusammenfassung. Es wurde die Wirkung von Nikotin und EDCT auf die spirakuläre Aktivität von Periplaneta americana (L.) untersucht. Nikotin hat eine hemmende und EDCT eine ausfrevende Wirkung auf das Gehirn. Untersuchungen an intakten, dezerebrierten und dekapitierten Insekten haben gezeigt, dass das Gehirn als Zentrum der Atmungshemmung wirkt. Versuche mit Gehirnxtrakten bei dekapitierten Insekten beweisen die hemmende Rolle des Gehirns und zeigen, dass ein unbekanntes Neurohormon für die spirakuläre Aktivität des Insektes von Bedeutung sein könnte.

S. S. Bhatia and G. T. Tonapi

Department of Zoology of the University, Poona 7 (India), 24 May 1968.

An Osmiophilic Substance in Brain Synaptic Vesicles not Associated with Catecholamine Content

Autoradiographic studies of catecholamine-storing nerve endings of the rat brain have indicated that despite retention of more than 50% of the radioactivity after glutaraldehyde-OsO₄ double fixation, no osmiophilic material is seen within the small synaptic vesicles (400 to 600 Å) by electron microscopy. Although larger vesicles (800–1000 Å) within the autoradiographically labeled nerve endings do exhibit osmiophilic granular material, the relative electron-opacity of the material does not fluctuate in proportion to monoamine content of the brain after pharmacological elevations or depletions of monoamine stores.

For peripheral autonomic nerves, the amount of electron-opaque material within synaptic vesicles correlates well with the catecholamine content of the nerves as judged by biochemical, fluorescent histochemical and autoradiographic assays. Glutaraldehyde pre-fixation appears to result in better demonstration of osmiophilic vesicular material than is seen with only OsO₄ fixation. Immersion fixation with cold 3% KMH₄O₄ is reported to result in the highest frequency of staining of the intravesicular material with sympathetic nerve endings. Brain fixed with KMH₄O₄ has also been reported to reveal an intravesicular material within brain synaptic vesicles of the rat median eminence and locus coeruleus. However, this fixative is difficult to use, since penetration of tissue is slow and tissues are difficult to section.

Since glutaraldehyde fixation of brain is associated with monoamine retention by autoradiography, but does not reveal electron-opacity in the small synaptic vesicles, the morphological discrepancy between the results of glutaraldehyde-OsO₄ fixation and KMH₄O₄ fixation do not seem to be directly explicable simply on the basis of better retention of vesicle catecholamine content. The present experiments have investigated the inability of glutaraldehyde-OsO₄ fixation to produce the synaptic vesicle electron-opacities revealed in the brain with KMH₄O₄: the vigor of reaction conditions has been varied to uncover possible physico-chemical differences in the reactivity of the 2 oxidants with intravesicular substances.

Materials and methods. Normal rats, rats pre-treated with reserpine (2.5 mg/kg, s.c., 18–25 h) or pargyline (100 mg/kg, i.p., 20 h) and rats given 35 μCi H₃-norepinephrine (sp. act. 6.5 Ci/mM) by injection into 1 lateral cerebral ventricle (2 h) were used. Brains were fixed by perfusion with 5% glutaraldehyde (phosphate-buffered, pH 7.4). Adjacent tissue blocks were exposed to solutions of 1% OsO₄ or 3% KMH₄O₄ for a variety of times and at different pH levels.

**Fig. 1.** Two nerve endings in paraventricular hypothalamus of normal rat, after fixation with glutaraldehyde and exposure to 1% OsO\textsubscript{4} at 60°C for 30 min. Synaptic vesicles in lower ending are filled with electron opaque osmiophilic precipitates, while vesicles in upper nerve ending are electron lucent. × 52,000.

**Fig. 2.** Neuropil of paraventricular hypothalamus from rat treated with reserpine (2.5 mg/kg, s.c., 24 h before). Heavy arrows indicate 4 nerve endings exhibiting the osmiophilic precipitates within synaptic vesicles. Thin arrows indicate 5 of the adjacent nerve endings with normally appearing electron lucent synaptic vesicles. Fixation and OsO\textsubscript{4} exposure identical to tissue illustrated in Figure 1. × 12,900.

different temperatures. After dehydration, blocks were embedded in Maraglass and thin sections were examined unstained in a Zeiss EM 9 electron microscope. Light microscopic autoradiographs were prepared from brains of animals given intraventricular H\textsuperscript{3}-norepinephrine, by dipping 4 μ Maraglass sections in Ilford L4 emulsion as previously described\textsuperscript{11}. Half of the blocks from radioactively labeled brains were exposed to OsO\textsubscript{4} at room temperature; the other half were exposed to 1% OsO\textsubscript{4} for 30 min at 60°C. Relative retained radioactivity was estimated by counting silver grains after exposures of 7–14 days.

**Results and discussion.** The fine structural examination was initially confined to the paraventricular hypothalamic region since this area is known to be rich in catecholamine-containing nerve endings\textsuperscript{12}, and since this area has been intensely studied by us previously\textsuperscript{1,4}. It was quite striking, therefore, to find that hypothalamic blocks exposed to 1% OsO\textsubscript{4} at 60°C for 30 min demonstrated nerve endings containing intravesicular osmiophilic granular precipitates (Figure 1). Such nerve endings constituted at least 40% of the nerve endings now found in this brain region. The maximum electron-opacification of synaptic vesicles occurred at a depth of 100–150 μ from the surface, at which point there was good electron contrast of plasma and intracellular membranes. At points closer to the outer surface of the block, contrast of membranes was also quite intense, but intracellular cytoplasmic material appeared eroded. Within the center of the block, electron-contrast was only slightly greater than normal, and synaptic vesicles exhibited no osmiophilic material. No treatment of the glutaraldehyde-fixed blocks with KMnO\textsubscript{4} produced any type of intravesicular opacities, although at higher temperatures, the tissue was completely dissolved.

In the hypothalamus of animals treated with either reserpine or pargyline, those nerve endings exhibiting intravesicular deposits had essentially the same relative number of reactive vesicles as normal animals (Figure 2). Further dissociation of the intravesicular osmiophilic material from tissue catecholamine content was established by examining autoradiographs of adjacent hypothalamic blocks from animals given intraventricular injections of H\textsuperscript{3}-norepinephrine\textsuperscript{12}. Those tissue blocks exposed to the warm OsO\textsubscript{4} treatment consistently exhibited less than \(\frac{1}{2}\) the number of grains seen over adjacent tissue from the same brains prepared with OsO\textsubscript{4} at room

\textsuperscript{12} B. Falck, Prog. Brain Res. 8, 28 (1964).