tion movements even when treated with fumigants, is described.

Nicotine when introduced in Ringer's solution flooding the insect's body[6] 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 neurohumor is involved in the spiracular regulation of respiration[7].

*Zusammenfassung.* Es wurde die Wirkung von Nikotin und EDCT auf die spirakulare Aktivität von *Periplaneta americana* (L.) untersucht. Nikotin hat eine hemmende und EDCT eine aufregende Wirkung auf das Gehirn. Untersuchungen an intakten, dezerebrierten und dekapitierten Insekten haben gezeigt, dass das Gehirn als Zentrum der Atmungsinhibition wirkt. Versuche mit Gehirnextrakten bei dekapitierten Insekten beweisen die hemmende Rolle des Gehirns und zeigen, dass ein unbekanntes Neurohormon für die spirakulare 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.*

[7] Acknowledgments are due to the Council of Scientific and Industrial Research, New Delhi, for providing a Junior Research Fellowship to one of us (S. S. B.). We are most indebted to Prof. W. Ebeling, University of California, Los Angeles, for reading the manuscript. Also thanks are due to the Head of the Department of Zoology and the University of Poona for facilities.

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

Autoradiographic studies of catecholamine-storing nerve endings of the rat brain[1,2] have indicated that despite retention of more than 50% of the radioactivity after glutaraldehyde-$OsO_4$ 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[3,4].

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[5–7] assays. Glutaraldehyde pre-fixation appears to result in better demonstration of osmiophilic vesicular material than is seen with only $OsO_4$ fixation[8,9]. Immersion fixation with cold 3% $KMnO_4$ is reported to result in the highest frequency of staining of the intravesicular material within sympathetic nerve endings[7]. Brain fixed with $KMnO_4$ has also been reported to reveal an intravesicular material within brain synaptic vesicles of the rat median eminence and locus coeruleus[10]. 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-$OsO_4$ fixation and $KMnO_4$ 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-$OsO_4$ fixation to produce the synaptic vesicle electron-opacities revealed in the brain with $KMnO_4$: 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 μC $H^3$-norepinephrine (sp. act. 6.5 C/m*M*) 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% $OsO_4$ or 3% $KMnO_4$ for a variety of times and at

[1] G. K. Aghajanian and F. E. Bloom, J. Pharmac. exp. Ther. *156*, 407 (1967).
[2] N. J. Lenn, Am. J. Anat. *120*, 377 (1967).
[3] K. Fuxe, T. Hokfelt and O. Nilsson, Am. J. Anat. *117*, 33 (1965).
[4] F. E. Bloom and G. K. Aghajanian, J. Pharmac. exp. Ther. *159*, 261 (1968).
[5] L. S. Van Orden, F. E. Bloom, R. J. Barrnett and N. J. Giarman, J. Pharmac. exp. Ther. *154*, 185 (1966).
[6] D. E. Wolfe, L. T. Potter, K. C. Richardson and J. Axelrod, Science *138*, 440 (1962).
[7] K. C. Richardson, Nature *210*, 756 (1966).
[8] F. E. Bloom and R. J. Barrnett, Nature *210*, 599 (1966).
[9] J. P. Tranzer and H. Thoenen, Experientia *23*, 123 (1967).
[10] T. Hokfelt, Z. Zellforsch. mikroskop. Anat. *79*, 110 (1967).

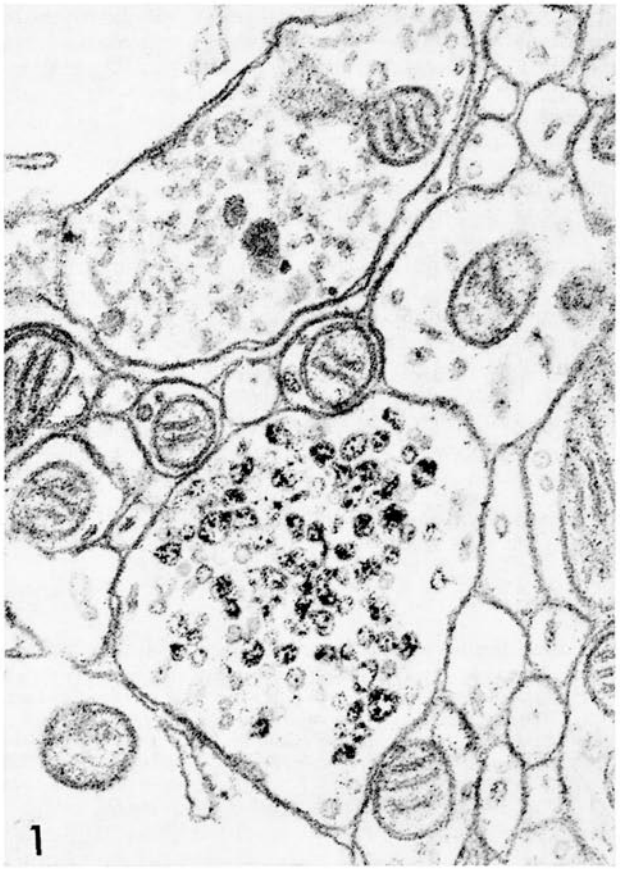


Fig. 1. Two nerve endings in paraventricular hypothalamus of normal rat, after fixation with glutaraldehyde and exposure to 1% $OsO_4$ at 60 °C for 30 min. Synaptic vesicles in lower ending are filled with electron opaque osmiophilic precipitates, while vesicles in upper nerve ending are electron lucent. × 52,000.

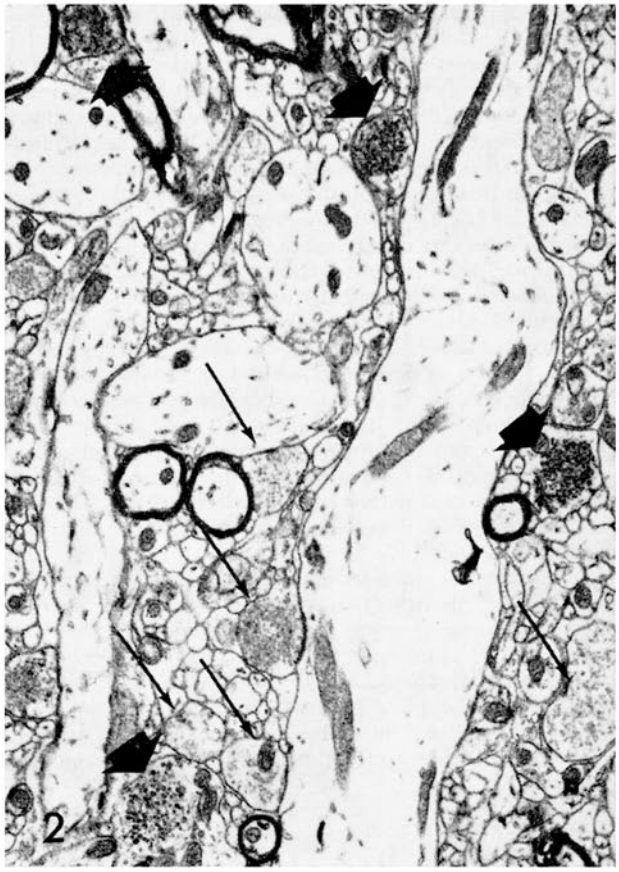


Fig. 2. Neuropil of paraventricular hypothalamus from rat treated with reserpine (2.5 mg/kg, s.c., 24 h before). Heavy arrows indicate 4 nerve endings exhibiting the osmiophilic precipitates within synaptic vesicles. Thin arrows indicate 5 of the adjacent nerve endings with normally appearing electron lucent synaptic vesicles. Fixation and $OsO_4$ exposure identical to tissue illustrated in Figure 1. × 12,500.

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 $H^3$-norepinephrine, by dipping 4 μ Maraglass sections in Ilford L4 emulsion as previously described[11]. Half of the blocks from radioactively labeled brains were exposed to $OsO_4$ at room temperature; the other half were exposed to 1% $OsO_4$ 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[12], and since this area has been intensely studied by us previously[1,4]. It was quite striking, therefore, to find that hypothalamic blocks exposed to 1% $OsO_4$ 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 $KMnO_4$ 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 $H^3$-norepinephrine[12]. Those tissue blocks exposed to the warm $OsO_4$ treatment consistently exhibited less than $^1/_2$ the number of grains seen over adjacent tissue from the same brains prepared with $OsO_4$ at room

[11] G. K. AGHAJANIAN, F. E. BLOOM, R. A. LOVELL, M. H. SHEARD and D. X. FREEDMAN, Biochem. Pharmac. *15*, 1401 (1966).
[12] B. FALCK, Prog. Brain Res. *8*, 28 (1964).

temperature, although the grain locations were always indicative of nerve ending labeling.

These results show that increased reactivity of the contents of certain types of brain synaptic vesicles toward $OsO_4$ can be obtained by performing the final fixation step at elevated temperatures. Although the product formed resembles superficially the granularity described within synaptic vesicles after $KMnO_4$ fixation, variation in catecholamine content occurs without detectable alteration in the appearance of the present osmiophilic substance. Moreover, the process which permits the visualization of this material actually seems to extract the catecholamine radioactivity. Thus, while granular synaptic vesicles may be an index to monoamine-storage, the granular material demonstrated by exposure to warm $OsO_4$ does not seem to be the monoamine itself. If the warming procedure facilitates the ability of $OsO_4$ to react with vesicle contents by increasing its potency as an oxidizing agent, then possibly other reducing substances[13] known to be present within the hypothalamus and related to monoamine neurons, such as ascorbic acid, could be responsible for the reaction observed here. The exact cause for the granular deposits developed by $KMnO_4$ fixation within presumed monoamine-containing nerves and its relation to the osmiophilic substance described here must now be investigated[14].

*Zusammenfassung.* Nach Fixierung durch Glutaraldehyddurchströmung und Behandlung mit $OsO_4$ bei 60 °C während 30 min erhält man im Rattenhirn elektronendichte Niederschläge innerhalb der kleinen synaptischen Vesikel. Amin-Entleerungsversuche und autoradiographische Untersuchungen haben gezeigt, dass diese Niederschläge nicht mit dem Katecholamingehalt des Gehirns zusammenhängen.

F. E. Bloom[15] and G. K. Aghajanian

*Departments of Psychiatry and Pharmacology, Yale University School of Medicine, New Haven (Connecticut, USA), 28 June 1968.*

[13] E. Makinnen and U. K. Rinne, Experientia *24*, 178 (1968).

[14] This research was supported by U.S.P.H.S. Grants No. MH 12380 and MH 11046.

[15] Present address: Laboratory of Neuropharmacology, NIMH, Washington (D.C. 20032, USA).

## Triphasic Intestinal Reaction on Adrenaline in the Rat

According to Ahlquist[1], Levy[2] and Furchgott[3] both canine ileum and rabbit duodenum relax after stimulation of either α- or β-receptors. Kawai[4,5], however, has found a contraction of guinea-pig ileum (maximal in the terminal part) after adrenaline (A). The present experiments in vitro were undertaken to analyse the effects of A upon rat intestinal segments.

*Results.* Adrenaline in concentrations of $5 \times 10^{-9} M$ to $1 \times 10^{-7} M$ caused relaxation and inhibited peristalsis in proportion to the amount of A in the bath. It is clearly evident that concentrations of $5 \times 10^{-7} M$ to $5 \times 10^{-6} M$ and sometimes $1 \times 10^{-5} M$ caused a triphasic change in the tone (Figure 1). After relaxation there followed an increase of the tone, which reached or overran the previous level, and was succeeded by a secondary relaxation. Atropine added to the bath fluid in concentration of 1 mcg/ml did not change these effects. The intensity of contractile phase was lower in duodenal segments and was increased in terminal parts of intestinal segments. The triphasic reaction to A in concentrations of $5 \times 10^{-7} M$ to $1 \times 10^{-5} M$ was also observed in rabbit terminal ileum and in human processus vermiformis tissue.

When propranolol in concentrations of $5 \times 10^{-5} M$ to $1 \times 10^{-4} M$ was added to the bath approximately 5 min before A, the relaxation of rat's intestine was prevented and the increase of tone was evidently augmented (compare 2 in Figure 1). Phentolamine in concentration of 5 mcg/ml inhibited the increase of tone observed after A, but either did not change or even increased the degree of relaxation (compare 3 in Figure 1 and C in Figure 2). The reaction of rat's intestine to high doses of A after phentolamine was similar to that observed after low doses of A. When both α- and β-blocking drugs were added to the bath concomitantly, A effects were completely prevented in concentrations up to $2 \times 10^{-5} M$. Methysergide maleate (deseril) added to the bath in concentration of $1–3 \times 10^{-3} M$ decreased slightly the intensity of triphasic reaction to A but did not change its character (B in Figure 2). Isoproterenol in concentration of $5 \times 10^{-9} M$ to $1 \times 10^{-6} M$ caused relaxation and disturbed peristaltic movements of rat's intestine and duodenum. Isoproterenol did not cause the contraction of intestinal smooth muscles, even in highest concentrations applied. The effects of isoproterenol were completely abolished by propranolol in concentrations of $5 \times 10^{-5} M$ to $1 \times 10^{-4} M$.

Since both isoproterenol and A applied in low concentrations caused relaxation of intestine, which was prevented by propranolol, it is evident that their action concerns β-receptors. However, the contractile phase of the reactions of intestine to high doses of A may be due to the stimulation of α-receptors, since it is prevented by phentolamine. The contractile phase of A effects does not change in the presence of propranolol, pointing to the same conclusion: adrenaline causes contraction and not relaxation of intestinal smooth muscles, exerting its influence by stimulation of α-receptors. Contractions of the gastrointestinal smooth muscles following α-receptor stimulation have been observed in muscle of the biliary tree of the guinea-pig by Crema et al.[6], and in both the

[1] R. P. Ahlquist and B. Levy, J. Pharmac. exp. Ther. *127*, 146 (1959).

[2] B. Levy, J. Pharmac. exp. Ther. *127*, 150 (1959).

[3] R. F. Furchgott, *Ciba Foundation Symposium on Adrenergic Mechanisms* (Ed. J. R. Vane, E. W. Wolstenholme and M. O'Connor; J. A. Churchill, London 1960), p. 256.

[4] A. Kawai, J. Osaka Med. Coll. *24*, 263 after Excerpta med., sect. 2c, *19*, 192 (1966).

[5] A. Kawai, J. Osaka Med. Coll. *24*, 271, after Excerpta med., sect. 2c, *19*, 192 (1966).

[6] A. Crema, G. Benzi, G. M. Frigi and F. Bertè, Proc. Soc. exp. Biol. Med. *120*, 158 (1965).