cell cultures were inoculated with diphtheria toxin dilutions. After 15 min the diphtheria toxin was eliminated using a syringe with a hypodermic needle and substituted with ATP or seroalbumin dilutions. Some cultures were treated with diphtheria toxin at the same concentrations but without any test substance, or with plain Hanks' BSS as a control.

Results. The effect of test substances is summarized in Table I. KCN prevents the swelling at all concentrations and for all toxin concentrations. Amytal and sodium azide are also very active preventing agents but they are ineffective for higher toxin concentrations. Rotenone gives an inconstant effect with the lower toxin concentration but it is completely ineffective using toxin concentrations higher than 1.0 Lf/ml (see Table I). ATP and seroalbumin promote actively the contraction of diphtheria toxin swollen mitochondria, but with higher toxin concentrations the mitochondria do no return to their filamentous shape and only a reduction in mitochondrial size can be observed (Table II).

Discussion. The findings reported in this paper show that the mitochondrial swelling induced by diphtheria toxin is an electron transport-dependent swelling and that this swelling is reversible, within certain limits, by ATP and seroalbumin. Anaerobiosis induced by KCN, Amytal, sodium azide and by other electron transport blocking agents inhibits the swelling induced by several substances in isolated liver mitochondria and the active electron transfer can be considered as a requisite for the swelling[4].

These findings support also the assumption that the toxin induced swelling is an active phenomenon. This concept is supported also by the action of ATP and seroalbumin which are related to mitochondrial contraction and promote the reversal of swelling induced by several substances in isolated mitochondria[5–7]. It is interesting to observe that ATP and seroalbumin are active on the diphtheria toxin induced swelling for toxin concentrations ranging between 0.5 Lf/ml and 5.0 Lf/ml. It is conceivable that, with higher toxin concentrations, an irreversible damage of the mitochondrial membranes occurs[8].

Riassunto. E stato studiato l'effetto di alcune sostanze sul rigonfiamento mitocondriale indotto dalla tossina difterica. Il KCN, l'Amytal e l'azide sodica prevengono il rigonfiamento. ATP e sieroalbumina ripristinano, entro certi limiti, la forma filamentosa in mitocondri rigonfiati dalla tossina difterica.

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Clinica delle Malattie Infettive, Università di Napoli, Ospedale Gesù e Maria, Napoli (Italy), 13th February 1967.

Table II. Reversal of swelling induced by ATP and seroalbumin in the mitochondria of diphteria toxin-treated cells (chicken embryo heart cells cultured in vitro). —, ineffective; + and ++, partial reversal of swelling; +++, complete reversal of swelling

Diphtheria toxin	ATP			Seroalbumin		
	10 μM	1 mM	10 mM	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml
0.5 Lf/ml	—	+++	+++	—	++	+++
1.0 Lf/ml	—	+++	+++	—	++	+++
5.0 Lf/ml	—	++	+++	—	+	++
10 Lf/ml	—	—	++	—	—	+
20 Lf/ml	—	—	++	–	–	+

[8] Acknowledgment: I wish to express my gratitude to Dr. G. F. PUCCINI, Dr. MCGUIRE and Dr. SVOBODA of the Eli Lilly Co. and Dr. W. GROPPI of the Wellcome Lab. for the generous supply of diphtheria toxin, and to Mr. R. GENTILE for his valuable technical collaboration.

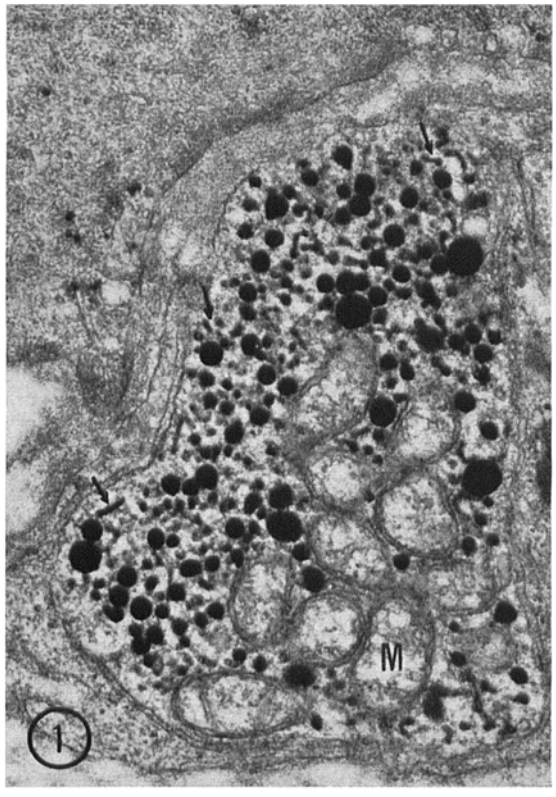
Electronmicroscopic Localization of 5-hydroxydopamine (3,4,5-trihydroxy-phenyl-ethylamine), a New 'False' Sympathetic Transmitter

In previous studies we have shown that pretreatment of cats with 5-hydroxydopa leads to a marked norepinephrine depletion and to an accumulation of 5-hydroxydopamine (5-HDA) in sympathetically innervated organs[1]. Concomitantly, the contractile response of the nictitating membrane and of the isolated perfused spleen to sympathetic nerve stimulation is greatly diminished, and 5-HDA is liberated as a transmitter into the splenic perfusion fluid.

It seemed to be of interest whether the replacement of the physiological transmitter norepinephrine (NE) by 5-HDA and its possible metabolites is accompanied by ultramorphological changes. Precipitation of 5-HDA with glutaraldehyde and the instaneous reduction of osmium tetroxide in the test tube seemed to provide favourable prerequisites for the electronmicroscopic localization of this new sympathetic transmitter substances.

Cats were given 4 × 20 mg/kg 5-HDA i.p. over a period of 48 h. Four h after the last dose, small pieces of iris, vas deferens, heart and spleen were removed, fixed in 3% glutaraldehyde (buffered at pH 7.4 with 0.1 M phosphate buffer), overfixed in 2% osmium tetroxide and embedded in epon for electronmicroscopic studies. The residual parts

[1] H. THOENEN, W. HAEFELY, K. F. GEY and A. HÜRLIMANN, Arch. exp. Path. Pharmak., in press.



of the organs were homogenized and their norepinephrine content was determined following the method of BERTLER et al.[2]. In all organs studied the NE content was reduced to less than 10% of that of untreated controls.

Ultrathin sections of the spleen capsule, which contains only adrenergic nerves[3], showed that all the vesicles in the autonomic nerve endings contained a large quantity of strong osmiophilic material. In comparison to non-treated controls, the vesicles seemed to be strongly overloaded and on the average they were somewhat larger. The dense material filled the vesicles completely and only at higher magnification was it possible to distinguish with certainty the limiting membrane. In addition, small cytoplasmic structures originating probably from the endoplasmic reticulum contained some dense material. The other ultrastructures of the nerves, e.g. mitochondria, microtubules, glycogen particles and the nerve limiting membrane, showed no detectable morphological alterations. Furthermore the smooth muscle cells, the basal

[2] A. BERTLER, A. CARLSSON and E. ROSENGREN, Acta physiol. scand. *44*, 273 (1958).
[3] H. THOENEN, J. P. TRANZER, A. HÜRLIMANN und W. HAEFELY, Helv. physiol. pharmac. Acta *24*, 229 (1966).

Fig. 1. Cat spleen capsule after treatment with 5-HDA. Autonomic nerve terminal containing vesicles and small cytoplasmic reticulum structures (→) filled with highly osmiophilic material. M mitochondria. × 50,000.

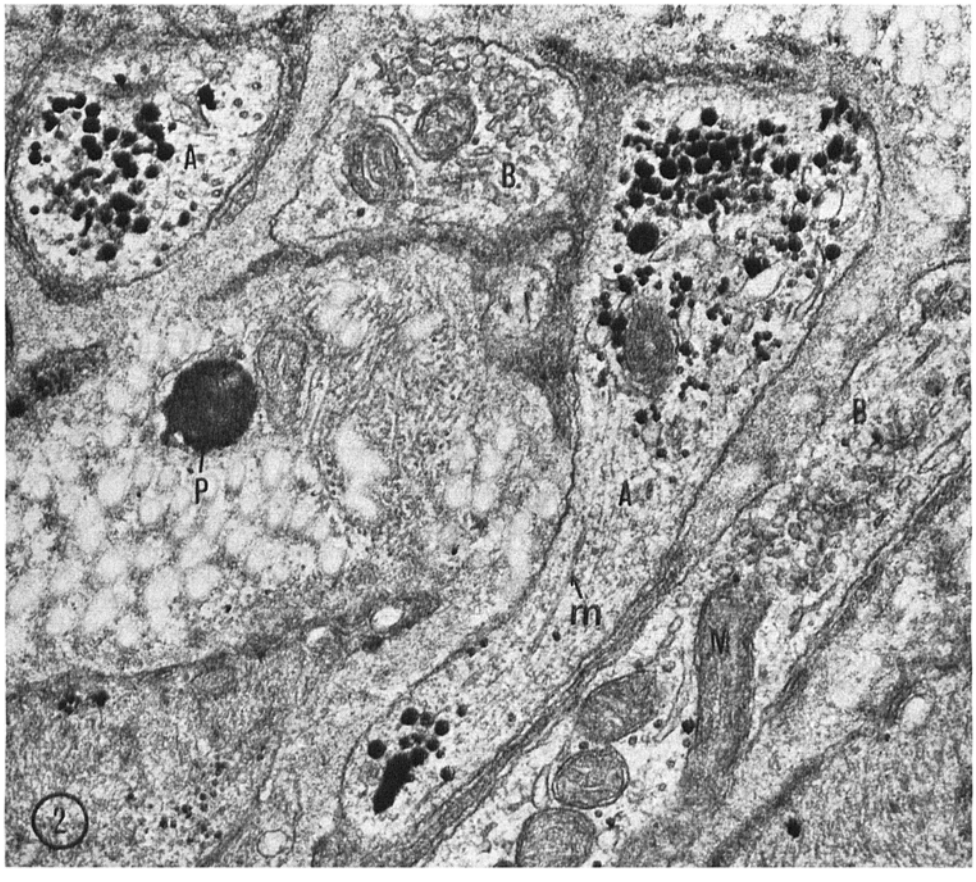


Fig. 2. Cat iris after treatment with 5-HDA. All vesicles of the adrenergic nerve sections (A) contain strongly osmiophilic material, whereas the vesicles of the cholinergic nerve sections (B) remain entirely empty. M, mitochondria; m, microtubuli; P, pigment. × 50,000.

membranes and the collagen fibres seemed normal and showed no increase in contrast.

In the iris and vas deferens where adrenergic and cholinergic fibres are often found in the same Schwann cell, some of the nerve terminals had the same appearance as in the spleen. Other terminals, probably cholinergic, contained exclusively empty vesicles (Figures 1–3).

The additional following findings support the assumption that the dense osmiophilic material accumulating in the vesicles of the adrenergic nerve terminals represents stored 5-HDA.

(a) A single i.v. injection of 10 mg/kg 5-HDA led to similar ultramorphological changes as early as 10 min after the injection.

(b) Incubation of thin slices of iris or spleen from non-treated cats in Krebs-Henseleit solution containing 1 mg/ml 5-HDA for 5–30 min gave analogous results.

(c) Treatment with 5-OH-Dopa (3 × 200 mg/kg i.p.) instead of 5-HDA caused similar ultramorphologic changes in adrenergic nerves of iris and spleen whereas the norepinephrine content of the corresponding organs was strongly reduced[1].

(d) In cats pretreated with α-methylmetatyrosine (α-MMT) 200 mg/kg, 20 and 4 h before the experiment, the autonomous nerve endings contained only empty vesicles in all organs studied. The NE-content was reduced to less than 5% of that of untreated controls. Incubation of iris and spleen slices of these animals in solutions containing 5-HDA led to findings similar to those in experiment (b) i.e. the adrenergic nerve endings contained strongly osmiophilic vesicles whereas the cholinergic ones all remained empty.

(e) A further similar experiment was performed after pretreatment with reserpine (2 mg/kg i.p. 20 h before experiment). In contrast to the pretreatment with α-MMT not only the cholinergic but also the adrenergic nerve endings remained empty after incubation in 5-HDA.

From these findings we conclude that the dense osmiophilic material filling the vesicles of the adrenergic nerve terminals represents 5-HDA and its possible β-hydroxylated and/or O-methylated metabolites (unpublished results) which have displaced the physiological transmitter norepinephrine.

The results of the 2 experiments with α-MMT and reserpine agree well with those performed with NE in place of 5-HDA[4]. 5-HDA like exogenous NE can be taken up and stored in the vesicles following replacement of their endogenous NE by metaraminol after pretreatment with α-MMT, but not after pretreatment with reserpine. The latter drug seems to block the uptake of this false transmitter into the storage granules as in the case of the physiological transmitter NE.

These drastic ultramorphological changes observed after pretreatment of cats with 5-HDA seem not to be irreversible, since 2 weeks after the last injection the ultramorphological aspect once more closely resembles that of normal tissues. Only a few abnormally filled vesicles can be seen at this time.

Previous experiments have provided strong evidence that empty and dense core vesicles in adrenergic nerve terminals represent a potentially uniform population of cell organelles differing only in their degree of amine filling[5]. The present experiments give additional strong support to this assumption, since after pretreatment with 5-HDA we were unable to detect any empty vesicles in adrenergic nerve terminals, whereas the vesicles of cholinergic nerves remained empty.

In conclusion the present investigation has shown that pretreatment of cats with 5-HDA, an amine acting as a 'false' sympathetic transmitter, leads to the accumulation of a highly osmiophilic substance in the vesicles of sympathetic nerve terminals, whereas their norepinephrine content is severely reduced. Strong evidence is offered that this dense material represents mainly 5-HDA. The vesicles of cholinergic nerve endings remain empty.

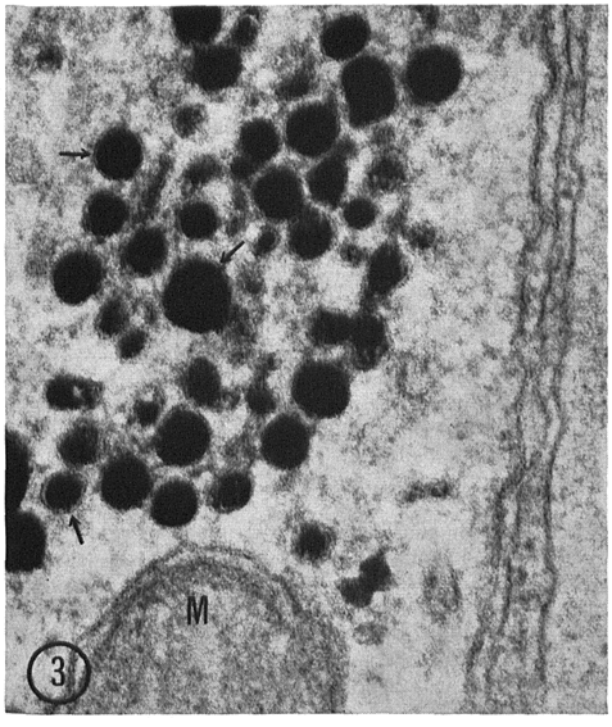


Fig. 3. Portion of an adrenergic nerve section at higher magnification. The limiting membranes of the vesicles are visible (→). The dense osmiophilic material fills the vesicles virtually completely. M, mitochondria. × 150,000.

Résumé. Le traitement de chats par la 5-hydroxydopamine (5-HDA), une amine agissant comme «faux» transmetteur sympathique, conduit à l'accumulation d'un matériel très osmiophile dans les vésicules des terminaisons sympathiques. Il a pu être démontré que ce matériel osmiophile représente principalement la 5-HDA. En même temps la teneur de la noradrénaline est fortement diminuée. Les vésicules des terminaisons nerveuses cholinergiques restent vides.

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