

## ENHANCEMENT OF THE GRANULATION OF ADRENERGIC STORAGE VESICLES IN DRUG-FREE SOLUTION

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Small granular vesicles within autonomic nerve terminals are now generally identified as storage sites for noradrenalin (Hökfelt, 1968). However, these granules do not always appear in the one preparation after different fixation procedures, and even with the same fixation technique they can occur in some adrenergically innervated tissues but not in others. It has been shown by Richardson (1966), and confirmed by others, that permanganate fixation reveals the granules, although the over-all fixation of the tissue is not usually as good with permanganate as with, for example, osmium tetroxide or glutaraldehyde. With osmium tetroxide fixation, little granulation of the vesicles of the adrenergic nerves in the rat iris has been found (Nilsson, 1964; Hökfelt and Nilsson, 1965), although granules have been found in the adrenergic axons of the vas deferens in the same species (Richardson, 1962). Furthermore, Merrillees et al. (1963), who duplicated Richardson's fixation methods but used

the guinea pig vas deferens, were unable to find granulation of the small vesicles of adrenergic axons. More recently, Watanabe (1969) examined the innervation of the guinea pig vas deferens and found granular vesicles after glutaraldehyde fixation but not after osmium tetroxide fixation. Similar difficulties have been found in revealing granules in adrenergic neurons of the central nervous system (Fuxe et al., 1965) and of the cerebral arteries (Iwayama et al., 1970), although they have been found in degenerating adrenergic nerves of the cerebral artery (Iwayama, 1970).

In the present work it is shown that incubation in Krebs' solution can profoundly influence the granulation of adrenergic storage vesicles in tissue fixed with osmium tetroxide.

### MATERIALS AND METHODS

Pieces of vas deferens from adult guinea pigs (weighing 150-400 g each) and rats (weighing 100-300 g each) were fixed immediately after sacrifice or following

immersion for up to 8 hr in modified Krebs' solution (Furness, 1970), maintained at 36°C, and bubbled with a mixture of O<sub>2</sub> (95%) and CO<sub>2</sub> (5%).

† The tissue was fixed in 1% osmium tetroxide in phosphate buffer (pH 7.3) for 1 hr. It was then washed in distilled water and placed in 4% phosphate-buffered glutaraldehyde (pH 7.3) for 1 hr followed, after washing, by a further hour in the osmium tetroxide fixative. The fixation method was suggested by Kanaseki, Uehara, and Imaizumi (unpublished). The tissue was stained in 2% uranyl acetate in distilled water for 1 hr and then dehydrated in acetone and embedded in Araldite. All of the procedures were carried out at room temperature. Thin sections of the embedded specimens were stained with lead citrate and examined under an Hitachi HU 11b electron microscope.

Pieces from the same preparations were prepared by the fluorescence histochemical method for demonstrating biogenic amines (Falck, 1962). The tissue was incubated in formaldehyde vapor at 80°C for 1 hr and embedded in paraffin, and 10 μ sections were taken for examination under ultraviolet light.

## RESULTS

The results from both rats and guinea pigs were essentially the same, and so, except where a distinction is made, the description applies for both species.

The localization of noradrenalin in the tissue was examined by fluorescence histochemistry prior to incubation and after periods of up to 8 hr in Krebs' solution. The definition and intensity of the fluorescence were not altered by the incubation; in all tissue, many bright fluorescent fibers were seen in both muscle coats. The fluorescent appearance of the adrenergic

nerves of the vas deferens after 8 hr in vitro is illustrated in Fig. 1.

Electron microscope examination of tissue fixed immediately after dissection showed very few granular vesicles in the axons of either muscle coat of the vas deferens (Figs. 2 and 3). In general, granulation of the small vesicles in the rat was only faint (Fig. 2) and few axon profiles contained granular vesicles; in the guinea pig almost no granulation was detectable (Fig. 3). In a very few cases, marked granulation of axonal vesicles of the unincubated vas deferens of the rat was observed.

In contrast, when the tissue was fixed after incubation in Krebs' solution, small, granular vesicles were consistently and prominently observed in many axon profiles (Figs. 5–8). Granular cores began to be clearly observed in axonal vesicles after 1 hr of incubation (Fig. 4) and became prominent in the axons of the circular muscle after 2–3 hr in vitro. The process of granulation was slower in the longitudinal than in the circular muscle. After 3–4 hr of incubation, most of the axon profiles of the inner muscle coat contained granular vesicles (Figs. 5 and 6). At 8 hr the granulation of vesicles in the axons of the circular muscle was very prominent (Fig. 7), while vesicles in the axons of the longitudinal musculature were still less heavily granulated (Fig. 8).

In general, the granulation of the vesicles was more intense and more frequent in rats than in guinea pigs (compare Figs. 5 and 6). Even when the granulation of many small vesicles was prominent, the small vesicles in some axon profiles were exclusively agranular (Fig. 6).

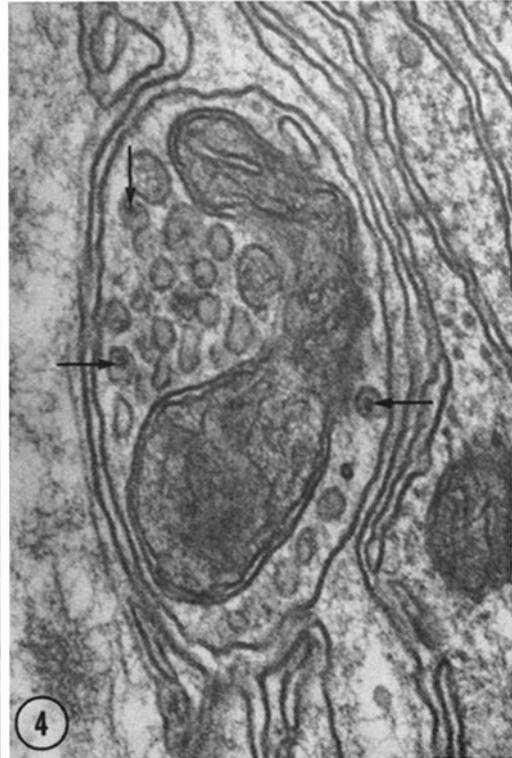
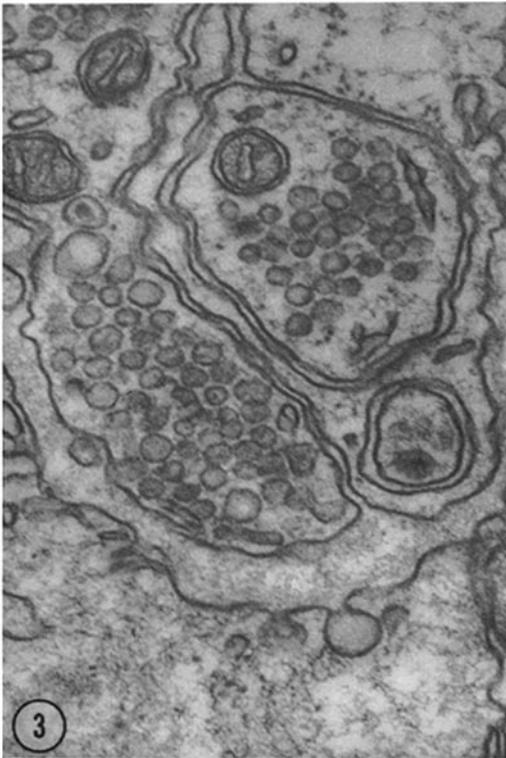
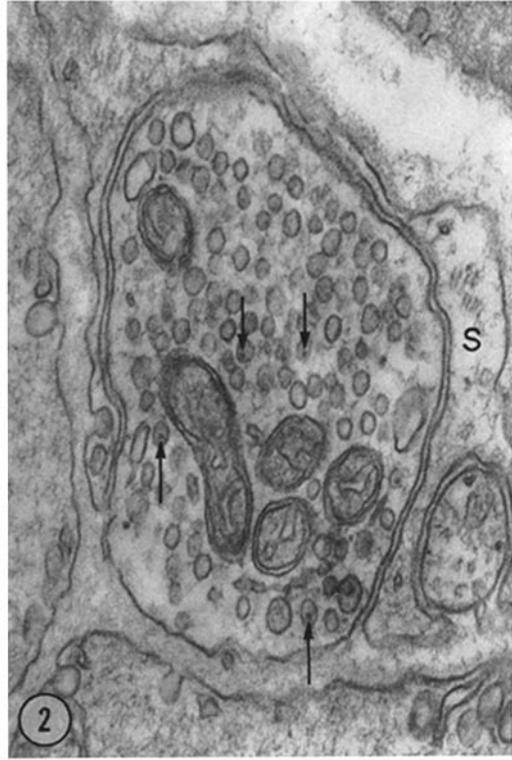
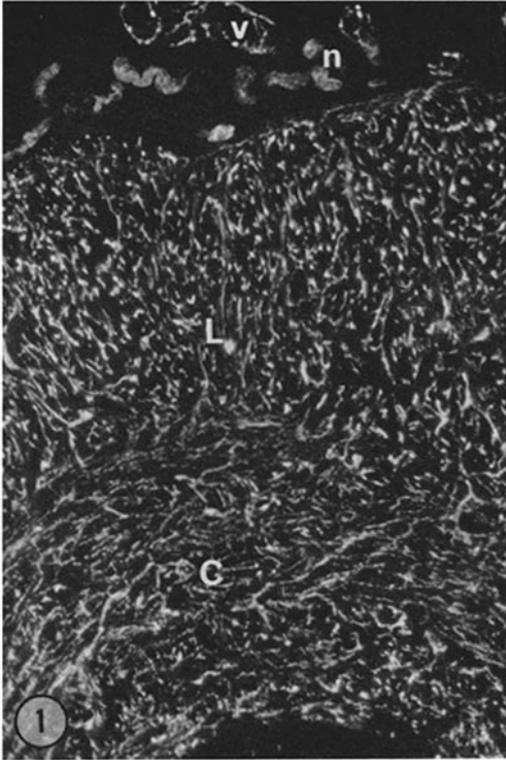
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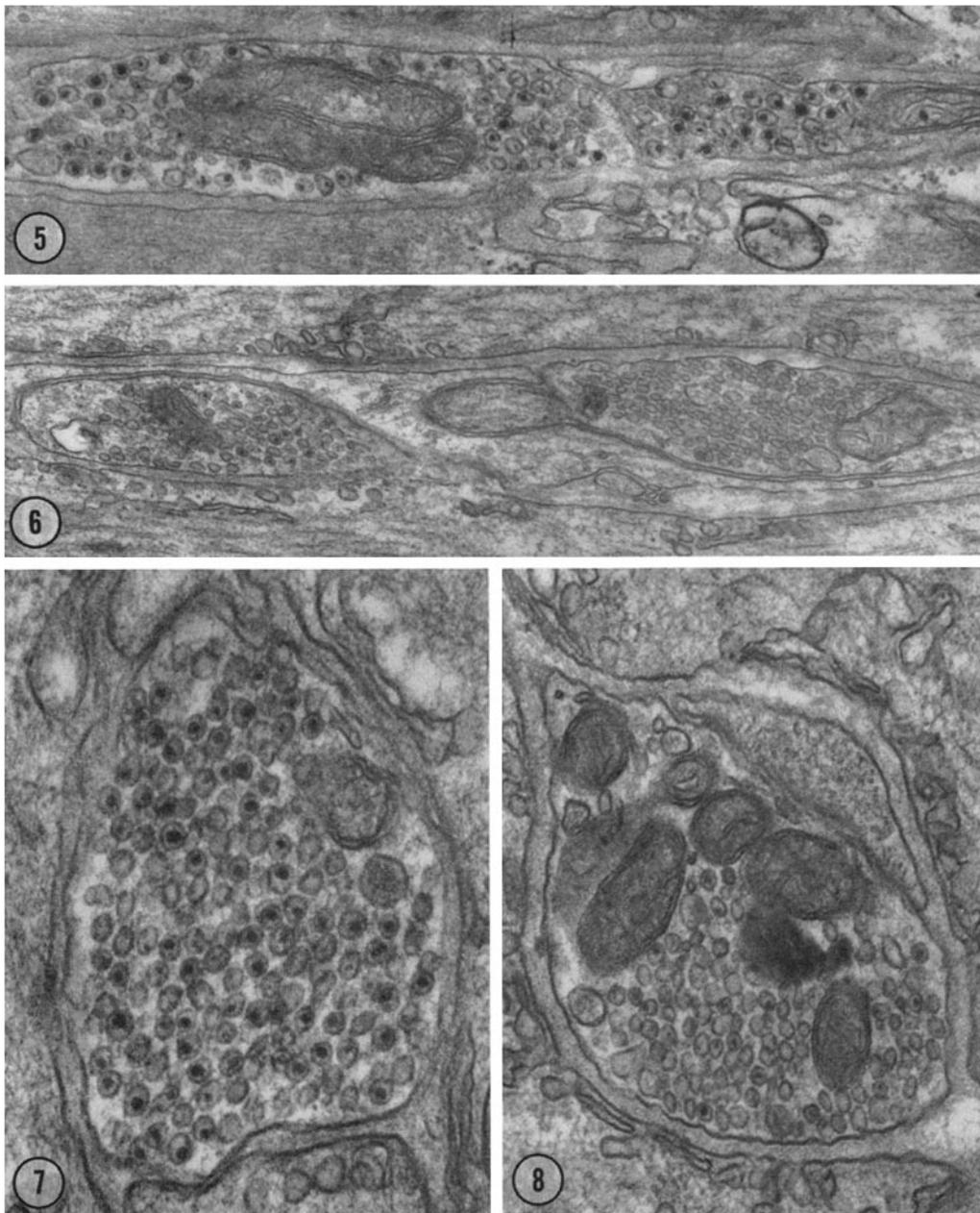
FIGURE 1 The adrenergic nerves in the guinea pig vas deferens demonstrated by the fluorescence histochemical method. The vas deferens was incubated for 8 hr at 36°C in Krebs' solution before being taken for examination. Innervated blood vessels (*v*) and small nerve trunks (*n*) can be seen at the surface of the vas deferens. Both the longitudinal (*L*) and circular (*C*) muscles are heavily innervated. × 140.

FIGURE 2 An axon profile from the inner part of the longitudinal muscle of the rat vas deferens fixed immediately after dissection. The axon is incompletely surrounded by Schwann cell (*S*) and contains many vesicles and some mitochondria. Most of the vesicles are about 500 Å in diameter. The matrix within the vesicle is slightly more electron opaque than is the axoplasm. Some vesicles contain small, faint granules (arrows). × 65,000.

FIGURE 3 A section from the circular muscle coat of the guinea pig vas deferens (not incubated). Two axon profiles containing small vesicles can be seen. No granules can be clearly observed in the vesicles. × 80,000.

FIGURE 4 An axon profile from the circular musculature of the guinea pig vas deferens. The tissue was incubated in Krebs' solution for 1 hr prior to fixation. At this stage, granules begin to appear in some of the small vesicles (arrows). × 115,000.





**FIGURE 5** Granulation of small axonal vesicles after 3 hr of incubation in Krebs' solution. This slender axon profile is from the circular muscle coat of the rat vas deferens. Note the variation in the size and shape of the granules.  $\times 42,000$ .

**FIGURE 6** Axon profiles of the circular muscle of the guinea pig vas deferens, fixed after 3 hr in Krebs' solution. One axon profile contains small granular vesicles, but the other contains small vesicles which are exclusively agranular.  $\times 28,000$ .

**FIGURE 7** Intense granulation of vesicles in an axon in the circular muscle of the rat vas deferens after incubation for 8 hr.  $\times 75,000$ .

**FIGURE 8** An axon profile of the longitudinal muscle of the guinea pig vas deferens after 8 hr in vitro. Many of the small vesicles are granular.  $\times 63,000$ .

## DISCUSSION

Small vesicles with electron-opaque cores are very difficult to detect after osmium tetroxide fixation of the untreated guinea pig vas deferens (Merrillees et al., 1963; Merrillees, 1968; Watanabe, 1969). However, in the present work, numerous small vesicles with prominent granulation were found in both rat and guinea pig vas deferens after incubation in Krebs' solution. This increase in the frequency and prominence of the granulation of adrenergic vesicles is not likely to reflect a comparable increase in transmitter content. Rather, it seems probable that the transmitter-protein complex in adrenergic vesicles undergoes some physicochemical change in vitro which alters its reaction with osmium. This change is possibly related to a deterioration of the axons which were severed from their cell bodies when the organ was removed. A transient increase in the granulation of small vesicles has been noted in adrenergic axons of the cerebral arteries during degeneration in vivo (Iwayama, 1970).

The results indicate that caution must be exercised in relating granulation to amine content, particularly in comparing control tissue to tissue which has been treated in vitro with drugs which act on amine stores. The enhancement of granulation which occurs in vitro may be strong enough to mask a decrease in amine content or to erroneously suggest an increase. Conditions of incubation which modify the appearance of adrenergic granules are being further investigated.

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