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F. STORAGE PARTICLES IN NORADRENERGIC TISSUES

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COMPARISON WITH ADRENAL MEDULLARY GRANULES

The existence of specific intracellular storage granules for catecholamines was first demonstrated for adrenal medullary tissue in 1953 (4, 27). During the following years a number of basic features of granule physiology were established, using the adrenal medullary granules as a model system. Unfortunately this has led to a wide-spread and unwarranted tendency to extrapolate from *in vitro* studies of adrenal medullary granules to the *in vivo* physiology of sympathetic nerves.

In 1956 Euler and Hillarp (15) demonstrated the existence of similar catecholamine-storing particles in bovine splenic nerves and in the rat spleen. The granules obtained from bovine splenic nerves, which turned out to be a convenient source, were found to have several features in common with the adrenal medullary granules. Thus they have a high ATP content, giving the same molar ratio of catecholamine to ATP of about 4:1 as the medullary granules (22, 39). The nerve granule membrane is freely permeable to catecholamines at 0°C, just as the medullary granule membrane is (45). The amine uptake mechanism in the nerve granules is potentiated by ATP (19) and inhibited by reserpine (20, 23), in agreement with previous observations with medullary granules.

However, the nerve granules have also been found to differ from the medullary granules in many respects. Thus they are smaller and more resistant to osmotic changes and to freezing and thawing than the medullary granules (16, 45). On incubation *in vitro* they are quite stable at 0°C but at higher temperatures they have a much higher spontaneous rate of amine release than the medullary granules, at 37°C leading to a 50 % depletion in about 10 min (16, 45). Certain drugs known to deplete the norepinephrine (NE) stores of sympathetically innervated tissues *in vivo*, like reserpine, surprisingly enough retard the NE release from the nerve granules, on incubation *in vitro* at low concentration (21). Direct comparison of the effects of such drugs *in vitro* on the spontaneous release of catecholamines and ATP from nerve and medullary granules shows distinct differences between the two types of granule (24). Perhaps most important of all, the catecholamine turnover rate, as determined by measurement of the rate of exchange of endogenous for exogenous catecholamine on incubation *in vitro* in the presence of tritiated *dl*-NE, is about 10 times higher in the nerve than in the medullary granules (45).

Thus it may be concluded that the medullary granules, which have served admirably for the studies of the basic principles of the physiology of catecholamine granules, in many respects distinctly differ from the nerve granules. Extrapolation from data obtained from medullary granules to the physiology of noradrenergic nerve terminals appears to be both hazardous and unnecessary, since the nerve granules are by now fairly well characterized and easily available.

COMPARISON WITH "ORGAN" GRANULES

The application of results obtained with the nerve granules isolated from the bovine splenic nerve trunks to the physiology of the sympathetic nerve terminals is based on the assumption that the nerve granules in different parts of the neuron have identical properties. Recent studies of the corresponding particulate fraction isolated from heart tissue in various species largely corroborate this assumption. Thus data obtained from experiments with rat heart granules (38) closely agree with observations made with bovine splenic nerve granules with respect to stability in various media, spontaneous amine release rate and molar ratio of catecholamine to ATP. In some respects, however, the heart granules were found to differ from the nerve granules. Thus the spontaneous amine release was not inhibited by reserpine, and the amine uptake was moderately potentiated by ATP and inhibited by reserpine only at excessively high concentrations. The striking absence of effect of reserpine, added to the incubation medium, on spontaneous rate of amine release has been confirmed for rabbit heart granules (14). However, reserpine administration *in vivo* strongly retarded the spontaneous amine release from these granules on subsequent incubation *in vitro* (14). The reason for this apparent discrepancy between the effects of this drug *in vitro* and *in vivo* remains unknown. In a comparative study of bovine adrenal medullary granules, bovine splenic nerve granules, and guinea pig heart granules (42), the three types of granule were found to be similar in several ways. However, the heart and nerve granules in a number of respects differed from the medullary granules. Perhaps the most significant difference was observed in the response to Ca^{++}, which accelerated the spontaneous rate of amine release in the medullary granules, while being completely devoid of effect on the nerve or heart granules (42).

The conclusion that all kinds of granule in postganglionic sympathetic neurons have identical properties, which differ from those of granules in chromaffin cells, is as yet premature. In fact, preliminary observations with granules isolated from the seminal vesicles of the bull support the possibility that granules from short noradrenergic neurons may in some respects resemble chromaffin cell granules rather than nerve granules (14).

BIOCHEMICAL CHARACTERISTICS

Several of the features characterizing the nerve granules were reviewed recently (by Euler at the Biogenic Amine Release Symposium in Stockholm, 1965), and will therefore not be dealt with here. Among the most interesting new aspects of the basic properties of these structures is the recent finding (26) that a mitochondria-free suspension of the high speed sediment from bovine splenic nerves has an active respiration. The positive proof that this activity occurs in "true" nerve granules has not yet been obtained. The enzymic set-up of these particles is at present under investigation.

ROLE OF GRANULES IN NE BIOSYNTHESIS

In current literature the nerve granules are frequently referred to as "storage particles." However, the granules also take part in the synthesis of NE. Thus

dopamine-β-hydroxylase activity has been demonstrated in rat heart granules (38), in accordance with the previous observation in medullary granules (30). In recent experiments the isolated bovine splenic nerve granules, suspended in isotonic potassium phosphate at pH 7.5, were shown to be able to form NE from dopa. This occurred with the nerve granule suspension obtained after the first low speed centrifugation, containing the original press juice from the nerve, as well as with granules resuspended in fresh potassium phosphate after a second centrifugation, and therefore contaminated with about $\frac{1}{350}$ of the original supernatant, and it occurred even with washed and resuspended granules, both with and without addition of pyridoxal phosphate and ATP-Mg^{++} (25). Similar incubation of nerve granules with tyrosine did not result in the formation of either dopamine or NE, either with or without addition of tetrahydropteridine as a cofactor for the tyrosine hydroxylase (36). The hydroxylation of tyrosine appears to require the presence of material removed from the nerve homogenate by the first low speed centrifugation. This is supported by the demonstration of dopamine and NE synthesis on incubation of the whole nerve homogenate with tyrosine (25, 26a). This suggests that the formation of dopa from tyrosine *in vivo* may occur during the passage of the latter through the axonal membrane, while the nerve granules carry out not only the last but the two last steps in the NE synthesis, the decarboxylation of dopa and the β-hydroxylation of dopamine.

The formation of NE from dopa on incubation of isolated nerve granules was inhibited by reserpine (25). This finding supports the concept that inhibition of NE synthesis may be one important mechanism of the maintenance of the NE-depletion caused by this drug (3, 31).

MECHANISM OF DRUG-INDUCED NE RELEASE

The term NE "releaser" is frequently used in the literature for various agents known to cause a net decrease in the NE content of the tissues. Sometimes the term is more closely defined by the statement that the "releaser" acts by displacement of NE from the nerve granules. This applies to several drugs, including sympathomimetic amines. Among these, tyramine has been shown to cause an acceleration of the spontaneous decrease in the NE content of the bovine splenic nerve granules, on incubation *in vitro* (17, 41). In experiments with bovine adrenal medullary granules the deficit in catecholamines after incubation with tyramine was reported to be stoichiometrically compensated for by uptake of tyramine (41). This was interpreted as a displacement effect. However, the effect of tyramine was absent at 0°C (25, 41). Moreover the NE-depleting effect of tyramine was almost completely inhibited by reserpine at low concentrations (18).

Thus if the spontaneous release of NE from the nerve granules was inhibited, by reserpine or low temperature, the added drug was unable to deplete the nerve granules. In view of this it seems unlikely that tyramine depletes the nerve granules by displacing NE. The data rather support the alternative that tyramine acts by competing for uptake into the granules with NE molecules spontaneously released. Many other NE "releasers" may similarly act by com-

petitively inhibiting re-uptake into the granules of NE spontaneously released from them. If this is correct, the rate of spontaneous release of NE from the nerve granules may be the rate-limiting factor in NE depletion caused by many different agents.

The implication of this is that spontaneous amine release is a prerequisite for amine uptake, as previously indicated by reserpine experiments (20). In this context it may be pointed out that the enigma of the NE-depleting effect of drugs like reserpine is the fact that these drugs actually strongly retard the spontaneous release of NE from isolated nerve granules. This is a paradox which has not received due attention in the current discussion, where the emphasis of the reserpine action at the granule level has been put on its inhibitory effect on amine uptake. The reason for this unfortunate shift in emphasis is quite clearly extrapolation from data concerning the *in vitro* reserpine effect on bovine adrenal medullary granules, where reserpine does not affect spontaneous amine release, but strongly inhibits amine uptake (7, 31).

SPECIFICITY OF NE UPTAKE MECHANISM

The NE uptake mechanism of the nerve granules distinctly prefers *l*- to *d*-NE, as judged from the much stronger inhibitory effect of unlabeled *l*- than of *d*-NE on the uptake of labeled *dl*-NE into the nerve granules on incubation *in vitro* (46). The preference of *l*-NE to *d*-NE appears to be of the order of 6 to 1. It may be significant that this figure is remarkably close to the corresponding "affinity" coefficient which can be calculated from data obtained on the NE uptake into the isolated perfused rat heart (28).

However, the amine uptake and storage mechanisms of the nerve granules appear to have a limited specificity, since compounds more or less closely related to NE, like sympathomimetic amines (35) and possibly even guanethidine (10), are taken up and retained in the granules, unchanged or after β-hydroxylation (35). It was recently shown that the distribution of exogenous NE and various phenylethylamines between the high speed sediment and the corresponding supernatant was different, 48% of the NE being recovered from the particulate fraction but only 38% of the dopamine and 25% of the octopamine (35). The conclusion drawn by these authors is that the catechol- and β-hydroxyl-groups are of decisive importance for binding in the nerve granules, a circumstance that explains why NE, which has both, is most avidly taken up and retained.

EXISTENCE OF NON-GRANULE-BOUND NE INTRANEURONALLY

One issue which has recently been much debated is the question whether the NE recovered from the high speed supernatant from various tissues corresponds to an extragranular pool of NE *in vivo*, or if it rather represents an artifact, due to leakage from the granules during the homogenization procedure. The recovery of NE in particle-bound form is clearly dependent on the technique used. Thus homogenization of bovine splenic nerve tissue with an Ultra-Turrax was recently found to yield a recovery of up to 60% of particle-bound NE, while squeezing the tissue, wrapped in a piece of gauze, between nylon rollers regu-

larly resulted in a recovery of about 30% in the high speed sediment (14). This obviously suggests the possibility that an even gentler homogenization precedure might lead to the recovery of 100% in the particulate fraction. However, it could also indicate that a certain, relatively fixed fraction of the total amount of particles in the tissue was highly sensitive to the disrupting effect of the squeezing procedure, possibly by adsorbtion to the gauze, while other particles were resistant in this respect. This would then possibly reflect the existence of two or more different types of nerve granule, which is in fact supported by electron microscopical evidence (38a). Moreover, the above mentioned difference in the recovery of various β-hydroxylated phenylethylamines from the high speed sediment strongly suggests the existence *in vivo* of some "pool" of intra-axonal, nongranule-bound amines (35).

There is of course general agreement that some NE exists extragranularly in the axons at least temporarily, since otherwise exogenous NE could never reach the granules. Further, extragranular NE does not imply free solution and distribution throughout the axoplasm and consequent exposure to destruction by monoamine oxidase (MAO), but NE protected in the axon by a different mechanism, possibly by lipid binding (12).

ROLE OF NERVE GRANULES FOR NEUROTRANSMITTER RELEASE

In accordance with the concept of quantal release of the cholinergic neurotransmitter (25a, 29), the possibility has been discussed that NE release from the sympathetic nerve terminals is also due to discharge of entire granules into the synaptic cleft (6).

For several reasons this appears unlikely. Thus, the intact nerve granules, when injected intravenously, are virtually inert (38). The total number of nerve granules in a varicosity can be estimated[1] to between 1000 and 8000 and is thus too low to allow discharge of whole granules on each nerve impulse. Even at a "resting" impulse frequency of 1 per sec all the granules in a varicosity would be used up within 15 min to 2 hr. At a maximum physiological impulse frequency of 10 per sec all the granules would be discharged within 1.5 to 13 min, even if only one granule would be discharged per impulse. Obviously this mechanism for neurotransmitter release would require a tremendous rate of synthesis, not only of the transmitter, but of the small "factories" which produce the transmitter as well.

It has also recently been proposed that the NE discharged from the axon by depolarization of the axonal membrane is immediately derived from nerve granules (7). This concept appears to be essentially based on two types of well documented observation. Firstly, there is considerable evidence that the return of the response to electrical nerve stimulation, lost after reserpine treatment, is closely correlated with the return of the capacity to accumulate exogenous NE *in vivo* in sympathetically innervated tissues generally (2), as well as in the high

[1] Calculation based on electron microscopical evidence indicating average diameter for nerve granules of 500–1000 Å, while the "double-cone" of the varicosity is 1 μ thick and 2 μ long (13, 34, 38a).

speed sediment from such tissues (45), and to accumulate exogenous amines in the adrenal medulla *in vivo* (9) and in adrenal medullary granules *in vitro* (32). Secondly, extensive morphological evidence supports the view that the neurotransmitter release on nerve stimulation occurs from the varicosities (33, 37). which are known to contain the majority of the nerve granules.

This concept requires that the local changes of the environment of the "outermost" granules in the varicosities, accompanying the depolarization of the axonal membrane, should be able to cause an actual release of NE from the granules.

As previously mentioned, a direct "releasing" effect on the granules appears unnecessary, in view of the very high spontaneous turnover rate directly observed *in vitro*, leading to a 50 % exchange of endogenous for exogenous NE in less than 10 min (45). Moreover, the nerve granules have been shown to be quite resistant to a number of changes in the environment, including variations in the Ca^{++} concentration (42). Thus it also appears unlikely that the changes in the intraneuronal milieu accompanying the depolarization of the axonal membrane should be able to affect the spontaneous NE release from the nerve granules. Moreover, there is evidence that the source of the NE released by nerve stimulation is quite small (1, 5, 11), and possibly reserpine-resistant (43). This makes it even more unlikely that the nerve granules should constitute the immediate source of the neurotransmitter.

The only structure which is definitely known to be profoundly changed by a propagated nerve impulse is the axonal membrane. It is presumably organized as an orderly sequence of protein-lipid-protein (or polysaccharide) (11a). Lipid material from sympathetic nerves has been shown to have a remarkable capacity to bind NE reversibly (12). It seems worthwhile to consider the possibility that NE, lipid-bound in the axonal membrane itself, or possibly contained in some "membrane-bound, vesiculated structure" (46); might represent the small NE pool from which the neurotransmitter release occurs on depolarization of the axonal membrane (44). This "transmitter pool" may possibly be assumed to discharge all of its NE each time the membrane is repolarized, but must then be assumed to recapture most of the NE during the subsequent repolarization. The inevitable gradual loss of NE would have to be compensated for by refilling from nerve granules topographically unrelated to the axonal membrane. This might constitute a reserpine-resistant transmitter pool, "trophically" highly dependent on the intra-axonal granules, and therefore quickly depleted when the granule function is severely disturbed, *e.g.*, by reserpine treatment. The role of the nerve granules would be to act as a rate-limiting factor in neurotransmitter release, providing the sympathetic terminals with the "inertia" necessary to avoid undue waste of transmitter by uncontrolled discharge, which, even without drug treatment, might lead to paralysis of neurotransmitter function.

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