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Secretion of Proteins (Chromogranin A and Dopamine <latex>β</latex>-Hydroxylase) from a Sympathetic Neuron

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Secretion of proteins (chromogranin A and dopamine β-hydroxylase) from a sympathetic neuron

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Biochemical studies on adrenal chromaffin granules have shown that these particles contain specific soluble proteins, the chromogranins, (see Kirshner & Kirshner 1971) and specific membrane proteins, the chromomembrins (see Winkler 1971). Although the functions of the main components of the chromogranins are not known, the ability to identify these proteins by biochemical and immunochemical methods led directly to the discovery of the release of chromogranins upon stimulation of the adrenal medulla (for reviews see Kirshner & Kirshner 1971; Smith & Winkler 1971) and to the identification of two of these proteins (chromogranin A and dopamine β-hydroxylase) in sympathetic nerves (see Livett, Geffen & Rush 1971). Both

Table 1. Proteins released into the splenic perfusate upon stimulation of the splenic nerve

animal	perfusion fluid	frequency of stimulation Hz	protein	method of identification	reference
calf	Tyrode's solution	30	dopamine β-hydroxylase	enzymic activity	De Potter *et al.* (1969*a*); Smith *et al.* (1970)
		30	chromogranin A	immunochemical	De Potter *et al.* (1969*a*); Smith *et al.* (1970)
cat	blood	10	[^{14}C]protein	radioactivity	Geffen & Livett (1968); Geffen, Livett & Rush (1970)
	Krebs–Ringer solution	30	dopamine β-hydroxylase	enzymic activity	Gewirtz & Kopin (1970)
dog	Tyrode's solution	30	dopamine β-hydroxylase	enzymic activity	De Potter *et al.* (1969*b*); Smith *et al.* (1970)
sheep	not stated	10	dopamine β-hydroxylase	immunochemical	Geffen, Livett & Rush (1969); Geffen *et al.* (1970)
			chromogranin A	immunochemical	Geffen *et al.* (1969, 1970)

these proteins are present in the noradrenergic vesicles of the splenic nerve (see Banks & Helle 1971; De Potter 1971) and upon lysis of the vesicles most of the chromogranin A (De Potter, Smith & de Schaepdryver 1970) and up to 20% of the dopamine β-hydroxylase (Hörtnagl, Hörtnagl & Winkler 1969; De Potter *et al.* 1970) become soluble. It is natural, therefore, to ask whether these proteins are released together with noradrenaline upon stimulation of the nerve.

During the last two years, three groups of workers have reported that dopamine β-hydroxylase can be detected in perfusates collected from the spleens of four species upon stimulation of the respective splenic nerves (see table 1). Similarly, material cross-reacting with antisera to chromogranin A has been found in perfusates from calf and sheep spleens (see table 1). In this brief survey (for a detailed review see Smith & Winkler 1971) the following questions will be asked: (i) What is the evidence that the protein released into the splenic perfusate originate from the terminals of the splenic nerve? (ii) How are the proteins released from the nerve terminals? (iii) What is the subcellular origin and site of synthesis of the proteins?

Evidence that dopamine β-hydroxylase and chromogranin A are released from the splenic nerve

The spleen is a complex organ and it should not be concluded, without further tests, that a substance released into the perfusate upon stimulation of the nerve has been secreted from the nerve terminals. Three other possible sites of origin are: first, extra-adrenal chromaffin cells; secondly, blood pooled in the organ; thirdly, unidentified sites sensitive to the neurotransmitter. In studies on the release of dopamine β-hydroxylase from dog and calf spleens, and on the release of chromogranin A from calf spleen, De Potter *et al.* (1969*a*, *b*) used pharmacological tests to exclude these non-neural sites of origin. These experiments have been described in detail elsewhere (Smith, De Potter, Moerman & de Schaepdryver 1970) and will only be summarized here.

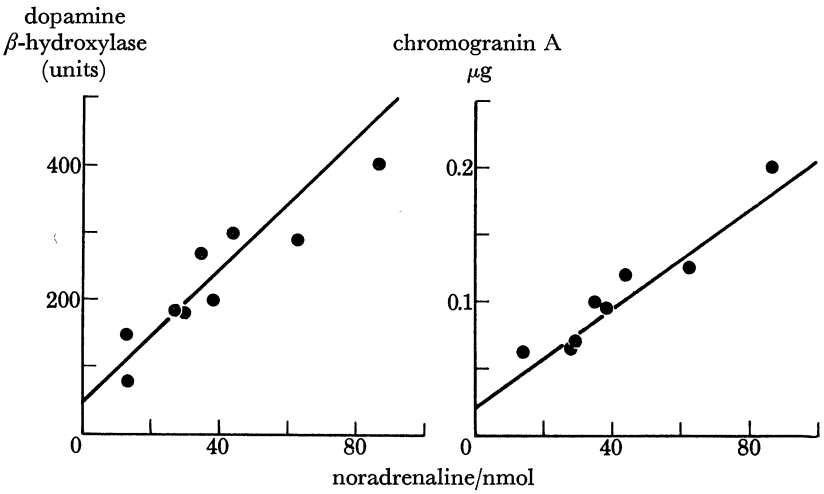

FIGURE 1. Correlation between the amounts of dopamine β-hydroxylase, chromogranin A and noradrenaline released from the calf spleen upon stimulation of the nerve. The correlation coefficient for dopamine β-hydroxylase and noradrenaline was 0.95, that for chromogranin A and noradrenaline was 0.92 (from Smith *et al.* 1970).

Stimulation of the splenic nerve releases acetylcholine, as well as noradrenaline, into the perfusate (Leaders & Dayrit 1965); the acetylcholine would stimulate any chromaffin cells that might be present in the spleen. However, when high concentrations of a ganglion-blocking agent (hexamethonium) were added to the perfusates in order to block the action of acetylcholine on chromaffin cells, stimulation of the splenic nerve still evoked the release of noradrenaline, dopamine β-hydroxylase and chromogranin A.

Since both chromogranin A and dopamine β-hydroxylase are released from the adrenal medulla, small amounts of these proteins might normally be present in blood. Although, in our experiments, the spleens were perfused with Tyrode's solution for 2 h before samples of the perfusate were analysed, some blood was still present in the tissue and was ejected into the perfusate when the spleen contracted. When an α-adrenergic blocking agent (phenoxybenzamine) was added to the perfusate, the spleen did not contract but the two proteins were still released.

The possibility that the neurotransmitter acts upon an unknown site in the spleen, causing the release of the proteins, was excluded by adding noradrenaline to the perfusion fluid.

Noradrenaline caused the spleen to contract, but did not bring about the release of dopamine β-hydroxylase.

These experiments show that the two proteins do not originate from chromaffin cells, from blood, or from unidentified sites in the spleen. Is it possible to obtain more direct evidence for the neural origin of these proteins? Since dopamine β-hydroxylase is an enzyme the properties of this protein can easily be studied. A comparison was, therefore, made between several properties of the dopamine β-hydroxylase in perfusates of dog and calf spleens, and the same properties of the enzyme in the soluble lysates of noradrenergic vesicles isolated from axons of the splenic nerve (Smith *et al.* 1970). No differences were found between the pH optima, mobilities in gel electrophoresis and sedimentation rates of the enzymes from the two sources; this is consistent with, but does not prove, the neural origin of the dopamine β-hydroxylase in the perfusate. More convincing evidence of the release of both dopamine β-hydroxylase and chromogranin A from the terminals of the splenic nerve, is the finding that there was an excellent correlation between the amounts of each protein released and the amounts of noradrenaline released (Smith *et al.* 1970): this is illustrated in figure 1. Since there is no doubt that noradrenaline is released from the nerve, we can conclude that dopamine β-hydroxylase and chromogranin A are released, together with the neurotransmitter, from the terminals of the splenic nerve.

How are the proteins released from the nerve terminals?

Studies on the composition of subcellular fractions of non-terminal axons of the splenic nerve (see De Potter 1971) have shown that dopamine β-hydroxylase and chromogranin A are components of the noradrenergic vesicles (large dense-cored type). If, as proposed below, the large dense-cored vesicles are the site from which the proteins are released in the nerve terminals, then we have to ask how these proteins pass into the extracellular space. Some of the possible mechanisms have been discussed by Kirshner & Kirshner (1971) in connexion with secretion from the adrenal medulla (see also Smith & Winkler 1971). Experiments designed to distinguish between these mechanisms have shown that secretion by exocytosis is the most likely mode of release from the adrenal medulla. Only a few such experiments have been done on the release of proteins from the splenic nerve: one question which has been studied is whether proteins in the soluble axoplasm are released. Dopa decarboxylase, an enzyme present in the soluble axoplasm of the splenic nerve (Stjärne & Lishajko 1967; De Potter *et al.* 1970), was not released into the perfusates when the splenic nerves of the dog or of the calf were stimulated (de Potter *et al.* 1969*a*, *b*). It has been calculated that if as little as 0.5 % of the noradrenaline was released by a process which increased the permeability of the nerve cell membrane to a protein molecule the size of dopa decarboxylase, it would have been possible to detect this by the radiochemical method of assay used for the enzyme (Smith *et al.* 1970). Furthermore, the sedimentation coefficient of dopa decarboxylase is less than that of the dopamine β-hydroxylase in the soluble lysate of noradrenergic vesicles (Smith *et al.* 1970) and so a molecule of dopa decarboxylase is probably smaller than a molecule of dopamine β-hydroxylase. Accordingly, the splenic nerve secretes a large molecule from a vesicle without at the same time releasing a smaller protein molecule from the axoplasm. This argues against a release mechanism in which the vesicle proteins are first released into the soluble axoplasm and then diffuse across the cell membrane. Exocytosis, however, provides an ideal way of secreting the contents of a vesicle without releasing components of the soluble axoplasm.

The secretion of the proteins by exocytosis is also consistent with observations that the

release of dopamine β-hydroxylase from the dog splenic nerve (De Potter *et al.* 1969*b*; Smith *et al.* 1970) and of both dopamine β-hydroxylase and chromogranin A (De Potter *et al.* 1969*a*; Smith *et al.* 1970) from the calf splenic nerve are dependent upon the presence of calcium in the perfusion fluid. (For a discussion of calcium and exocytosis see reviews by Douglas (1968), Matthews (1970) and Smith (1971)).

That exocytosis may indeed occur in sympathetic nerve terminals is indicated by electron microscopical studies, which show the fusion of the membrane of large dense-cord vesicles with the nerve-cell membrane in the vas deferens (Farrell 1968; Fillenz 1971). Fillenz (1971) has also given evidence of the fusion of the membrane of small dense-cored vesicles with the cell membrane. Exocytosis is seen only very rarely by the microscopist, but this does not necessarily mean that it is a rare event. Sometimes it is possible to assess the significance of exocytosis as a release mechanism by counting the number of 'exocytotic profiles' in micrographs of stimulated nerves, as was done for the corpus cardiacum by Normann (1969). Alternatively, as in the studies on the adrenal medulla, biochemical methods of analysis can be applied. The ratio of the amount of the chromogranins to the amount of catecholamine in adrenal perfusates was very close to this ratio in the soluble lysate of chromaffin granules (see Kirshner & Kirshner 1971) and so it can be concluded that most, if not all, of the catecholamine released from the adrenal medulla is secreted by exocytosis. Is it possible to decide what proportion of the noradrenaline released from the splenic nerve is secreted by exocytosis by a similar comparison? Before this question can be answered, we must know from which type of noradrenergic vesicle the proteins are secreted.

What is the subcellular site of origin and of synthesis of the secreted proteins?

Whereas the chromaffin granules of the adrenal medulla form a relatively homogeneous population of particles (for qualification of this see the review by Smith & Winkler (1971)), the noradrenergic vesicles of sympathetic nerves fall into two main types (see Fillenz 1971). Large dense-cored vesicles are found in cell bodies and in non-terminal axons as well as in terminal varicosities, while the small dense-cored vesicles are characteristic of the terminals. The morphological distinction between two types of noradrenergic vesicle is supported by biochemical studies, which have shown that nerve terminals contain two kinds of vesicle but that non-terminal axons contain only one type (Roth, Stjärne, Bloom & Giarman 1968; De Potter 1971). It is probable that the 'light' vesicles of Roth *et al.* (1968) are the small dense-cored vesicles, and that the 'heavy' vesicles are the large dense-cored vesicles (Bisby & Fillenz 1970). Although it is known that the large dense-cored vesicles of non-terminal axons contain ATP, chromogranin A and dopamine β-hydroxylase, our knowledge of the composition of the large and small noradrenergic vesicles of nerve terminals is less certain. In the terminals of the splenic nerve, the large dense-cored vesicles contain dopamine β-hydroxylase, but it appears that the small noradrenergic vesicles contain little, if any, of this enzyme (De Potter 1971). Studies on the small noradrenergic vesicles of the nerve terminals in the heart (Potter 1967) and vas deferens (M. Bisby, M. Fillenz & A. D. Smith, unpublished observations) have shown that dopamine β-hydroxylase is probably present in this type of vesicle. In the small noradrenergic vesicles from the heart, the dopamine β-hydroxylase has been reported to be entirely membrane-bound (Potter 1967).

Until more is known about the composition of the small noradrenergic vesicles, we must adopt a working hypothesis (Smith 1970; Smith *et al.* 1970); this is that the large dense-cored vesicles release the chromogranins, together with noradrenaline, by exocytosis, whereas the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

small dense-cored vesicles release only noradrenaline (see figure 2). In order to see what proportion of the noradrenaline released from the large dense-cored vesicles is secreted by exocytosis, we have to know the ratio of dopamine β-hydroxylase activity to the amount of noradrenaline in the soluble lysate of the large noradrenergic vesicles present in the nerve terminals. This ratio has been determined for the soluble lysates of the large noradrenergic vesicles from *non-terminal* axons of the splenic nerves of calf, cat and dog, and the values are given in table 2. However, it has recently been found that the large noradrenergic vesicles in the *terminals* of the dog splenic nerve are relatively enriched in noradrenaline: the ratio of

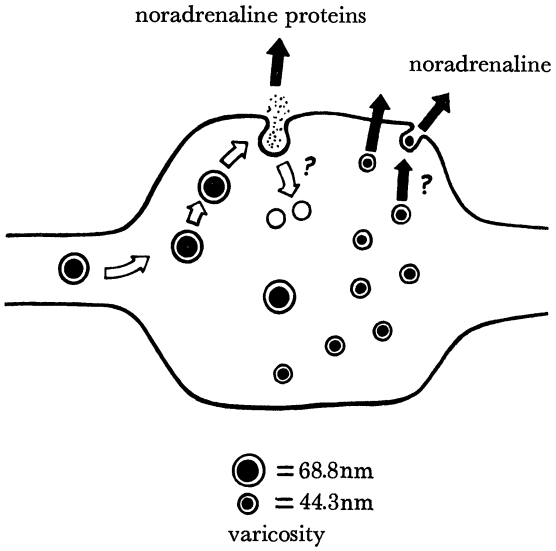

FIGURE 2. Diagram representing the types of noradrenergic vesicle in the splenic nerve to illustrate a hypothesis concerning the origin of the noradrenaline and proteins released from the nerve terminals. The large dense-cored vesicles, which are formed in the cell body and which migrate along the axon to the terminals, contain soluble proteins (chromogranin A and dopamine β-hydroxylase) in addition to noradrenaline. It is suggested that the small dense-cored vesicles, which may originate at the terminal, do not contain these soluble proteins. Stimulation of the nerve causes the release of noradrenaline from both types of vesicle and of the soluble proteins from the large dense-cored vesicles. The most likely mode of release of the contents of the large vesicles is exocytosis. The membrane of the large vesicle may then fragment to give smaller (? synaptic) vesicles as it pinches off the nerve cell membrane. The measurements of the sizes of the vesicles are taken from the paper by Geffen & Ostberg (1969) and refer to the splenic nerve of the cat. (Part of fig. 8 in the paper by Smith *et al.* 1970).

dopamine β-hydroxylase activity to noradrenaline in these vesicles is about one-tenth of that in the vesicles in non-terminal axons (see De Potter 1971). This interesting finding, perhaps related to the more ready availability of amino acid precursors of noradrenaline in the terminals, gives us the second column of figures in table 2. The third column of figures in table 2 are the experimentally determined ratios of dopamine β-hydroxylase activity to noradrenaline in perfusates from spleens of the calf, cat and dog: these ratios are 64, 76 and 33 %, respectively, of the corresponding ratios in the soluble lysates of the large noradrenergic vesicles in the nerve terminals (Similar calculations show that the ratio of chromogranin A to noradrenaline in calf spleen perfusates is about 9 % of that in the soluble lysate of large dense-cored vesicles: this finding is discussed by Smith *et al.* (1970).)

There are, of course, other factors to be taken into consideration. First, the amount of noradrenaline in the perfusate will be less than that released from the nerve due to uptake of the transmitter. Experiments using an inhibitor of the uptake, phenoxybenzamine, indicated that

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

about twice as much noradrenaline was actually released from the dog and calf splenic nerves as was recovered in the perfusate (Smith *et al.* 1970): this means that the ratios of dopamine β-hydroxylase to noradrenaline in the perfusates should be halved. Secondly, we do not know what proportion of the noradrenaline released from the splenic nerve comes from the small noradrenergic vesicles. In the terminals of the dog splenic nerve, about 40 % of the particulate noradrenaline is found in the small noradrenergic vesicles (De Potter 1971). If about half of the

TABLE 2. RATIOS OF DOPAMINE β-HYDROXYLASE ACTIVITY TO NORADRENALINE IN SOLUBLE LYSATES OF NORADRENERGIC VESICLE FROM SPLENIC NERVE, AND IN PERFUSATES COLLECTED FROM THE SPLEEN DURING STIMULATION OF THE NERVE

animal	ratio in noradrenergic vesicles of non-terminal axons	ratio in noradrenergic vesicles (large) of terminals	perfusate	references
dog	96	9.6	3.2	De Potter (1971); Smith *et al.* (1970)
calf	106	(10.6)†	6.8	De Potter *et al.* (1969*a*); Smith *et al.* (1970)
cat	427	(42.7)†	29	Gewirtz & Kopin (1970)

The ratios are in picomoles of octopamine formed in 20 min per nanomole of noradrenaline. The amounts of dopamine β-hydroxylase and noradrenaline released upon stimulation of the nerve were calculated by subtracting the amounts in perfusates collected before stimulation from those present in perfusates collected during stimulation.

† These values have been calculated on the assumption that the tenfold difference between the ratio of dopamine β hydroxylase activity to noradrenaline in the large noradrenergic vesicles of dog splenic nerve terminals and this ratio in the non-terminal vesicles (De Potter 1971), also applies to the calf and cat splenic nerves.

noradrenaline released from the nerve comes from the small vesicles, then the ratios of dopamine β-hydroxylase activity to noradrenaline in the perfusate must be doubled in order to compare them with the ratios in the soluble lysates of the large noradrenergic vesicles. The net effect of these two corrections is to leave the ratios in the perfusates more or less unchanged! As they stand, the ratios given in table 2 indicate that the amounts of dopamine β-hydroxylase in the perfusates are consistent with the release of a considerable part of the noradrenaline from the large dense-cored vesicles by exocytosis. The strength of this conclusion depends upon the validity of the assumptions made in the argument: fortunately, several of these assumptions can be tested experimentally by analysis of the composition of the two kinds of noradrenergic vesicle in nerve terminals.

Synthesis of the proteins

The final question to be discussed concerns the site of synthesis in the neuron of the proteins that are secreted from the terminals. The cell body is the major site of protein synthesis in neurons (Droz 1969; Barondes 1969). Proteins released from the terminals have, therefore, probably been synthesized in the cell body and transported along the axon to the terminals. The dopamine β-hydroxylase and chromogranin A of the non-terminal axons of the splenic nerve are stored in large dense-cored vesicles, and it has been found that noradrenaline and both the proteins migrate proximodistally along the axon (see Livett *et al.* 1971) and that the large dense-cored vesicles also migrate in the same direction (see Banks & Helle 1971). These findings are consistent with the idea that proteins which are to be secreted are synthesized in the cell body and transported in vesicles to the nerve terminals (Scott 1905).

Geffen & Ostberg (1969) suggested that the large dense-cored vesicles in the splenic nerve might be precursors of the small dense-cored vesicles that are found in the nerve terminals.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Such a transformation could come about by fission of intact large vesicles, in which case the ratio of dopamine β-hydroxylase to noradrenaline should be about the same for both types of vesicle. If the vesicles are formed in this way, it is difficult to see why the fission of large vesicles should occur only in the terminals. The terminals are, however, the site of release of the contents of the large vesicles by exocytosis. This leads to another suggestion (Smith 1970): the small noradrenergic vesicles might be formed by fission of the empty membranes of the large vesicles during retrieval of the membrane after exocytosis. (Morphological evidence of such a mechanism of membrane retrieval in the adrenal medulla has been given by Grynszpan-Winograd (1971).) In this case, the ratio of dopamine β-hydroxylase activity to noradrenaline should be lower in the small vesicles than in the large vesicles, since the small vesicles will only contain the membrane-bound dopamine β-hydroxylase. Studies of the noradrenergic vesicles from nerve terminals in the spleen (De Potter 1971) and in the vas deferens (M. Bisby, M. Fillenz & A. D. Smith, unpublished observations) have shown that the small vesicles do indeed contain less dopamine β-hydroxylase activity relative to their content of noradrenaline than do the large vesicles. Furthermore, it has been reported that the dopamine β-hydroxylase in the small vesicles from nerve terminals in the heart is entirely membrane-bound (Potter 1967). These preliminary observations are consistent with our working hypothesis that the proteins are secreted from the large noradrenergic vesicles.

What, then, is the function of the small noradrenergic vesicles which seem to be formed locally in the terminals? These vesicles may be primarily concerned with the local uptake, synthesis, and release of the neurotransmitter. The amount of noradrenaline in the large vesicles which migrate down the axon is small, and does not make a significant contribution to the function of the terminals (Geffen & Rush 1968). The transport of noradrenaline by the large vesicles is just incidental to the main functions of these vesicles, which seem to be: first, to supply the nerve terminals with the enzyme that converts dopamine into noradrenaline, secondly, to supply the nerve terminal with the specific membranes of the synaptic vesicles and, thirdly, to carry from the cell body to the terminals, those proteins which are destined to be secreted.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES