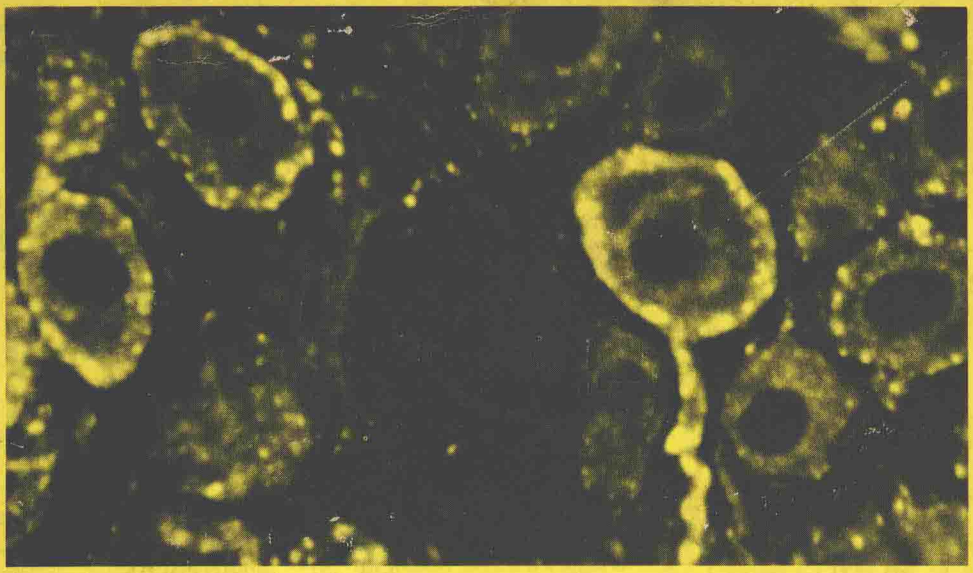
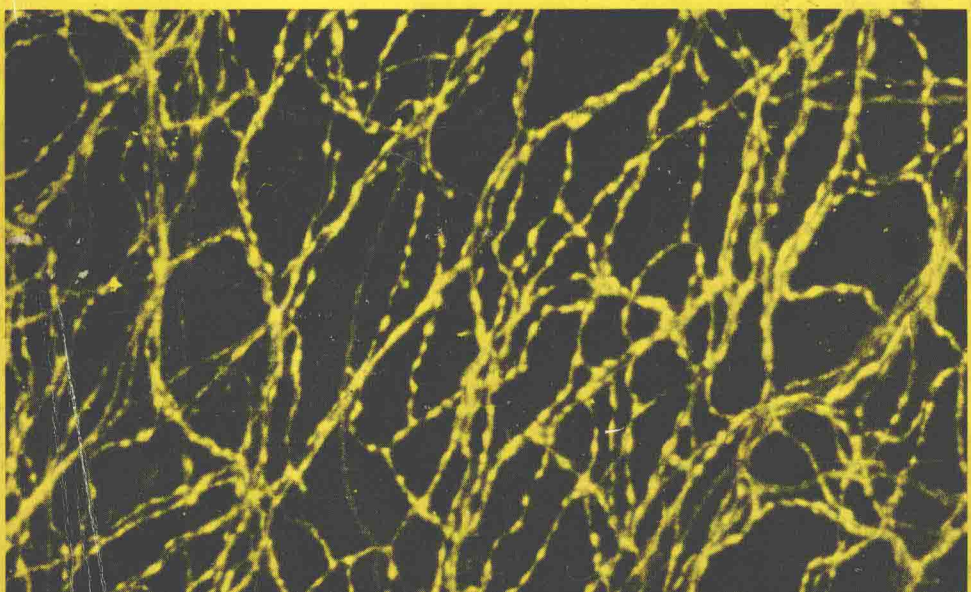


Studies of Neurotransmitters at the Synaptic Level

Edited by
E. Costa, L. L. Iversen and R. Paoletti

Advances in Biochemical Psychopharmacology 6



STUDIES OF NEUROTRANSMITTERS AT THE SYNAPTIC LEVEL

Advances in Biochemical Psychopharmacology Volume 6

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Volume 6

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Preface

During the past few years there has been rapid development in neurochemistry, particularly as it relates to neurotransmitters. The contributions described in this volume indicate the level of sophistication that this area of research has attained. The breadth and ingenuity of experimental approaches to the study of the life history of neurotransmitters is impressive. Electron microscopy, histochemistry, cell biology, pharmacology, enzymology, and chemistry have each made special contributions. The use of such a variety of disciplines has proven to be a powerful stimulus for rapid advances in our understanding of the biochemistry and pharmacology of neurotransmitters.

The neuron contains highly branched nerve terminals with specific pre- and postsynaptic uptake and receptor mechanisms. The nerve terminals also contain storage vesicles and enzymes that synthesize and metabolize transmitter substances. These enzymes have a different subcellular localization and are under many controls. In addition, the central nervous system has a varied regional composition of transmitters, cell bodies, nerve terminals, and enzymes. All of this makes the chemistry of the nervous system different from any other organ. Thus, many of the papers described in this volume deal with the regional chemistry at the synaptic level. Some also give a glimpse of the future of this area, the disposition of possible new neurotransmitters, and the physical isolation of postsynaptic receptors.

Julius Axelrod
Washington, D.C.

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Application of Cytochemical Techniques to the Study of Suspected Transmitter Substances in the Nervous System

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An increasing number of substances are suspected of acting as neurotransmitters in the mammalian central nervous system (see e.g. Hebb, 1970). In order to establish their transmitter nature, several criteria must be fulfilled (Florey, 1960; McLennan, 1963; Werman, 1966), one of which is the demonstration—preferably both at the light- and electron-microscopic levels—of the occurrence of the substance within the neuron from which it is suspected of being released upon the arrival of nerve impulses. This has so far been possible only for a group of biogenic monoamines including dopamine (DA), noradrenaline (NA), and 5-hydroxytryptamine (5-HT), thanks to the development and application of various techniques (for references see Bloom, 1972; Hökfelt and Ljungdahl, 1972) such as the Falck-Hillarp formaldehyde fluorescence method (Falck, Hillarp, Thieme, and Torp, 1962).

In this chapter, some aspects of the intraneuronal localization of the monoamines and their storage sites will be discussed and, against this background, some recent autoradiographic results on the cellular and fine structural localization of several suspected amino acid neurotransmitters will be presented. For a more systematic presentation of the topic, we refer the reader to two recent articles (Bloom, 1972; Hökfelt and Ljungdahl, 1972).

GENERAL ASPECTS ON APPLICATION OF TECHNIQUES

The ideal way to study histochemically the localization of suspected neurotransmitters is to involve the substance itself in a specific, chemical reaction *in situ*, i.e., at the "physiological" storage site in the tissue, resulting in a product visible in a microscope. This principle of a *direct* visualiza-

tion of the neurotransmitter has been achieved with the Falck-Hillarp method, where the amines react with formaldehyde vapors to form a fluorescent compound (Falck et al., 1962; Corrodi and Jonsson, 1967; Falck and Moore, 1972). In all probability, the ultrastructural demonstration of an electron-dense core (precipitate) within synaptic vesicles in monoamine neurons after various fixation methods (OsO_4, a combined glutaraldehyde-OsO_4 sequence, or $KMnO_4$) may represent similarly direct techniques for electron-microscopic studies (for discussion see Bloom, 1970, 1972; Hökfelt, 1970, 1971). Also in autoradiographic studies of suspected neurotransmitters (see, e.g., Wolfe, Potter, Richardson, and Axelrod, 1962; Aghajanian and Bloom, 1966; Descarries and Droz, 1970) the transmitter substance itself is traced. Future developments of new direct techniques may involve immunological principles – immunohistochemistry – since it has recently been possible to make antibodies to a small molecule such as histamine (Davies and Meade, 1970), one of the suspect neurotransmitters.

A different approach is based on attempts to visualize substances closely related to the suspected transmitter such as the enzymes involved in transmitter metabolism. This *indirect* tracing of transmitters may be examplified by the precipitation techniques for acetylcholine esterase (Koelle and Friedenwald, 1949) or choline acetyltransferase (Burt, 1970; Kása, Mann, and Hebb, 1970) for tracing neurons releasing acetylcholine (ACh) at the synapse. Immunohistochemical methods have also been introduced recently, but so far only for enzymes involved in catecholamine (CA) synthesis (Geffen, Livett, and Rush, 1969; Fuxe, Goldstein, Hökfelt, and Joh, 1970*a*, 1971; Goldstein, Fuxe, Hökfelt, and Joh, 1971; Hartman and Udenfriend, 1970). Enzymes involved in ACh and γ-aminobutyric acid (GABA) metabolism have also been traced autoradiographically using labeled enzyme inhibitors such as diisopropyl fluorophosphate (Ostrowski and Barnard, 1961), hemicholinium, and thiosemicarbazide (Knyihár and Csillik, 1970).

BIOGENIC MONOAMINES (DA, NA, AND 5-HT)

Methodology

The wealth of information available on the localization of DA, NA, and 5-HT both in nervous and non-nervous tissues stems from a variety of light- and electron-microscopic techniques (see Pearse, 1960). However, a more systematic mapping of the occurrence of monoamines in nervous tissues started out with the introduction of the Falck-Hillarp fluorescence

method—based on the formation of fluorescent isoquinolines (from DA and NA) or carbolines (from 5-HT) after paraformaldehyde treatment (see Corrodi and Jonsson, 1967)—leading to a profound knowledge of the distribution of monoamine neurons both in the peripheral and central nervous systems (see Fuxe, Hökfelt, Jonsson, and Ungerstedt, 1970*b*; Falck and Moore, 1972).

The fluorescence histochemical results have provided a valuable basis for attempts to extend the knowledge of localization of monoamines to the ultrastructural level. Such attempts to visualize a substance in the electron microscope are basically dependent on the possibility of involving the amine in a reaction with a heavy metal that will result in an electron-dense precipitate. Fortunately, test-tube experiments have revealed that DA, NA, and 5-HT all react with almost all routine fixatives for electron microscopy to form precipitates (see Table 1). As to one-step fixation procedures with

TABLE 1. *Reaction between various fixatives and amines in test-tube experiments*

Amine	Fixative				
	OsO_4[a]	$KMnO_4$[b]	PF[c]	Glut.[d]	Glut. + OsO_4[d] or $K_2Cr_2O_7$
Noradrenaline	+	++	0	+	+
Dopamine	+	++	0	+	+
Adrenaline	+	++	0	0	
5-Hydroxytryptamine	+	++	0	+	+
L-DOPA	+	++			
Metaraminol	0	+			
β-Phenylethylamine		0			

(From Hökfelt, 1971.)
The formation of a precipitate is indicated by a plus.
[a] Van Orden et al., 1966.
[b] Hökfelt and Jonsson, 1968.
[c] Hopsu and Mäkinen, 1966.
[d] Wood and Barrnett, 1964; Coupland et al., 1964.

metallic oxidants such as OsO_4 and $KMnO_4$, a redox reaction seems to take place, where the amines reduce the fixatives mainly to osmium dioxide and manganese dioxide, respectively. The hydroxyl groups of the amine molecules, mainly the phenolic ones, seem to be responsible for this reaction since NA (with two phenolic OH groups) gives a strong reaction, metaraminol (with one phenolic OH group) gives only a weak reaction, and β-phenylethylamine (with no OH group) gives no reaction when mixed in test tubes with $KMnO_4$ (see Fig. 1). Precipitates are also obtained in test tubes with a

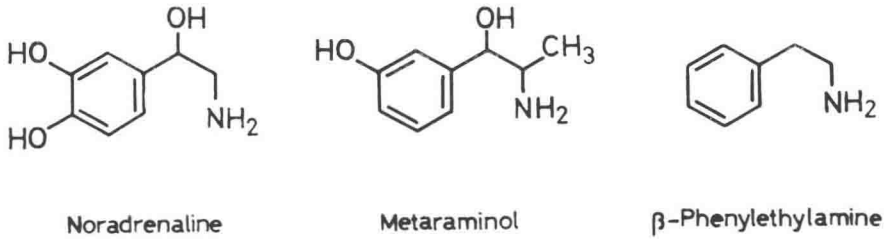


FIG. 1. Molecular structures of three monoamine analogs: noradrenaline (with two phenolic hydroxyl groups), metaraminol (with one hydroxyl group) and β-phenylethylamine (with no hydroxyl group). See text and Table 1.

glutaraldehyde-OsO_4 sequence. In the first step, glutaraldehyde probably reacts with the amine group (see, e.g., Tramezzani, Chiocchio, and Wassermann, 1964; Coupland, Pyper, and Hopwood, 1964; Wood and Barrnett, 1964) leaving the OH groups free for a subsequent reaction with OsO_4 as discussed above.

The first evidence from experiments on intact tissues that neuronal monoamines may react with the fixative and give rise to an electron-dense precipitate was obtained already in 1961, when De Robertis and Pellegrino de Iraldi (1961*a*, *b*) and Lever and Esterhuizen (1961) described a special type of synaptic vesicles containing an electron-dense precipitate (dense-core or granular vesicles) in probable adrenergic neurons. In subsequent studies on various tissues and species, these findings could sometimes be confirmed, sometimes not. Since then, owing to the introduction of new fixation procedures and pharmacological manipulations (some important advances are listed in Table 2), general agreement now exists that the so-called small granular or dense-core vesicles (see below) are monoamine storage sites (Fig. 2).

Although many pharmacological and experimental results favor the view that the dense core of the small granular vesicles reflects the amine content, there is some doubt about the validity of this correlation, at least for $KMnO_4$-fixed tissues (see, e.g., Bloom, 1972). This is mainly due to a lack of correlation between the presence of the electron-dense core and the retention of the amine as revealed in experiments with labeled NA (Devine and Laverty, 1968; Hökfelt and Jonsson, 1968). Thus, $KMnO_4$ fixation gives a high yield of dense-core vesicles (and few empty or agranular vesicles) but a low retention of the amine, whereas a combined fixation with glutaraldehyde and OsO_4 often gives few dense-core vesicles (and many empty vesicles) but a high retention of the amines (Table 3). This has re-

TABLE 2. *Fixation procedures for the demonstration of small granular vesicles in monoamine neurons*

Fixative	Tissue	Reference
A. Metallic oxidants		
1. OsO_4	Pancreas	Lever and Esterhuizen (1961)
	Pineal	De Robertis and Pellegrino de Iraldi (1961*a*)
	Vas deferens	Richardson (1962)
	Iris	Richardson (1964)
	Intestine	Grillo and Palay (1962)
2. $KMnO_4$	PNS, CNS	Richardson (1966)
		Hökfelt (1968)
B. Aldehyde + metallic oxidants		
1. Glut. ald.-OsO_4	Vas deferens	Bloom and Barrnett (1966); Van Orden et al. (1966)
	Pineal	Machado (1967)
2. Glut. ald.-$K_2Cr_2O_7$ ($-OsO_4$)	CNS	Wood (1966)
	Pineal	Jaim-Etcheverry and Zieher (1968)
	Iris	Tranzer and Thoenen (1968)
3. Glut. ald. + formald. -$K_2Cr_2O_7$($-OsO_4$)	Vas deferens	Tranzer and Snipes (1968)
4. Acrylic ald. -$K_2Cr_2O_7$($-OsO_4$)	Heart	Woods (1969)
5. Formald.-glut. ald. -$K_2Cr_2O_7$($-OsO_4$)	CNS	Wood (1967)
	Pineal (5-HT)	Jaim-Etcheverry and Zieher (1968)
C. Pharmacological tools increasing amine levels		
1. Endogenous, e.g., MAO inhibition		
2. Exogenous		
a) "True" transmitter, e.g., NA		Tranzer and Thoenen (1967*b*)
		Hökfelt (1968)
b) "False" transmitter, α-methyl-NA		Bondareff (1966)
		Hökfelt (1967, 1968)
5-OH-DA		Tranzer and Thoenen (1967*a*)

(From Hökfelt, 1971.)

sulted in difficulties with autoradiographic studies on $KMnO_4$-fixed tissues (Taxi, 1968; Bloom, 1970, 1972; Descarries and Droz, 1970).

The specificity of $KMnO_4$ fixation has been discussed previously (Hökfelt and Jonsson, 1968; Hökfelt, 1970, 1971), but the following points may be summarized.

(1) As seen in Table 3, all the amine is not lost but about 50% remains in the tissue, and autoradiographic studies in our laboratory have indeed shown that marked accumulations of grains can be seen, e.g., over nerve

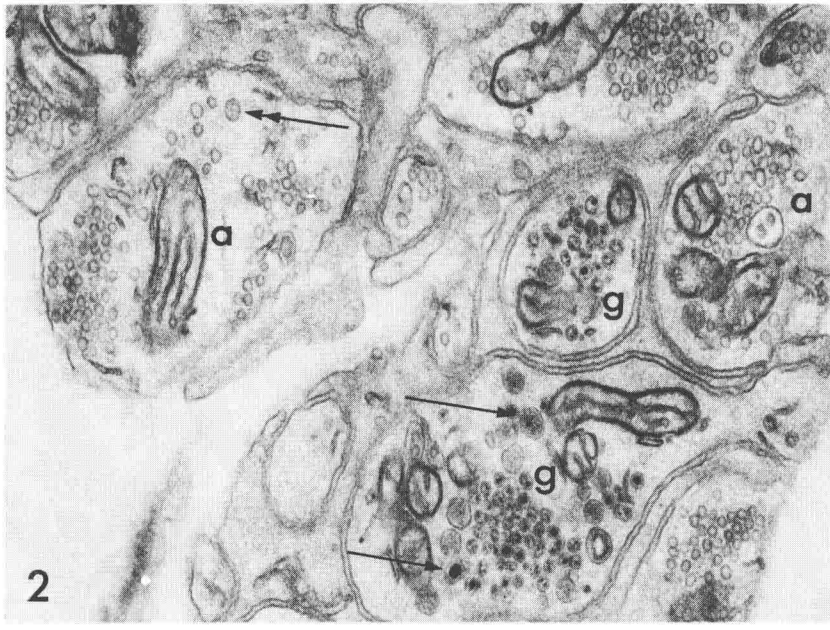


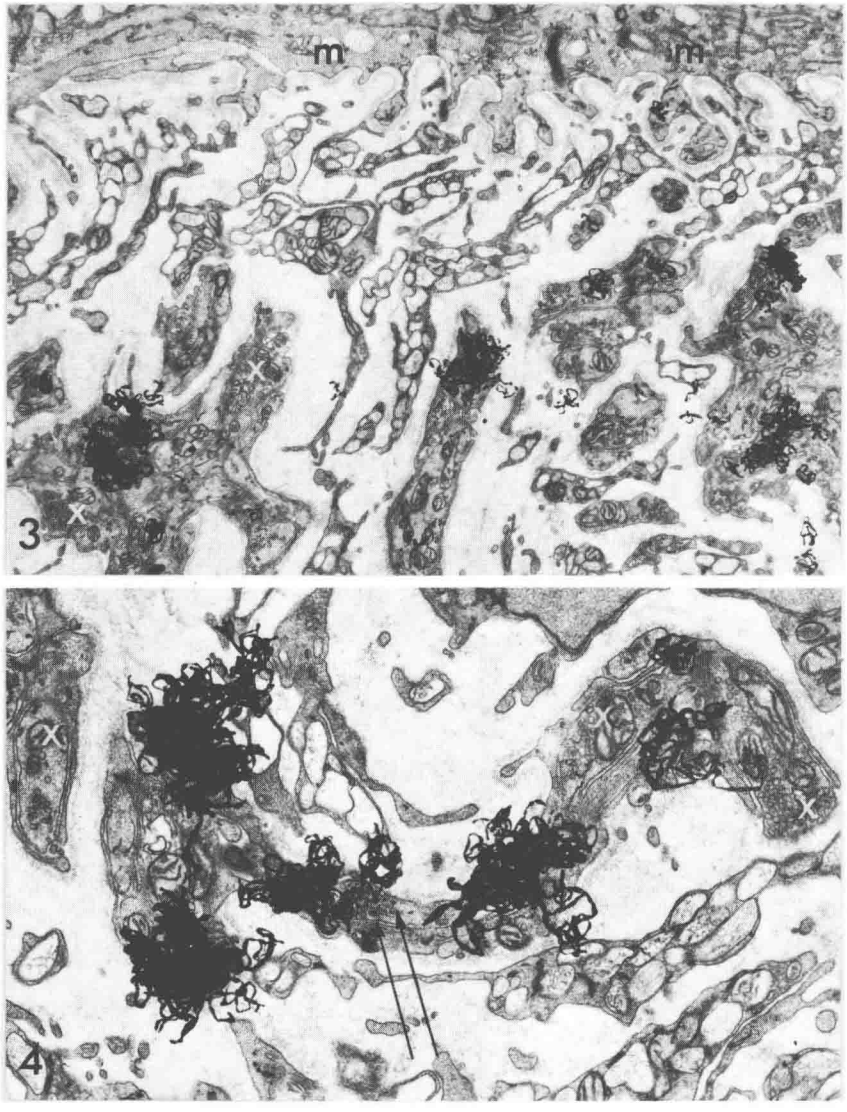
FIG. 2. Electron micrograph of guinea pig iris. A number of varicosities (nerve endings, axonal enlargements) are seen. Two (g), probably adrenergic ones, contain granular vesicles (mainly small ones) with a few of the large type (arrows). The other varicosities (a) contain only agranular vesicles—also, the large ones have an empty interior (double/arrow). They probably belong to cholinergic nerves. $KMnO_4$ fixation. Magnification × 35,000.

TABLE 3. *Fate of ^{3}H-NA during fixation and dehydration procedure*

	3% $KMnO_4$ (30 min)	5% Glut. (90 min)	5% Glut. (30 min)	1% OsO_4 (90 min)	5% Glut. (30 min) +1% OsO_4 (60 min)
Fixative I	18[a]	26	15	26	13
Ringer (10 min)	14	10	11	4	9
Fixative II	–	–	–	–	9
Ringer (10 min)	–	–	–	–	2
Ethanol (45 min)	2	7	4	1	0
Propyleneoxide (30 min)	0	0	0	0	0
Tissue	53	57	70	65	71
Total	87	100	100	96	104

(From Hökfelt and Jonsson, 1968.)

[a]The figures represent the percentage of radioactivity found in solutions and tissue as compared to the total amount of ^{3}H-NA present in control irides not fixed and processed.



FIGS. 3 and 4. Electron-microscopic autoradiographs of rat iris, incubated with ^{3}H-noradrenaline (10^{-6} M). A number of nerves are seen covered by strong accumulations of grains. The grains almost completely cover the varicosities but granular vesicles can be seen at arrows in Fig. 4. Nerve endings with agranular vesicles (x), however, lack activity. Muscle cells (m) of the dilator muscle plate are seen at the top of Fig. 3. $KMnO_4$ fixation. Magnifications × 10,000 and 20,000, respectively.

endings in the rat iris after incubation with ^{3}H-NA (10^{-6} M) and $KMnO_4$ fixation (Figs. 3 and 4).

(2) A low retention of amine after fixation does not imply a lack of correlation between precipitate and amine levels. Thus, after the initial redox reaction, the oxidized amine may not be chemically bound within the precipitate and may thus partially be washed out. It is, therefore, possible to state only that the dense core reflects the amine content *at the moment of fixation*, which is sufficient for most studies, with the exception, of course, of autoradiography.

(3) The specificity of the $KMnO_4$ fixation can be seriously questioned since $KMnO_4$ reacts with innumerable substances. Therefore, the correlation between precipitate and amines is not based on a specificity of the reaction between these two substances but rather on the fact that the vesicles contain the amines in extremely high concentrations, as the following calculation shows. The amount of NA in one varicosity of the adrenergic axon terminals in the rat iris dilator muscle has been calculated to be about 5×10^{-3} pg (Dahlström, Häggendal, and Hökfelt, 1966) and the number of vesicles in one varicosity to be roughly 500 (Hökfelt, 1969; see Fig. 5). The radius of one vesicle is about 250 Å of which the membrane constitutes 70 Å; i.e., the radius of the vesicle "cavity" is about 180 Å. If, hypothetically, all the NA is stored within the vesicles [i.e., if none is localized in the extravesicular space and none within the membrane (which in fact makes up about 65% of the total vesicle volume)], the NA concentration in the vesicle is about 2.4 M (Fig. 6)!

(4) In addition to these general considerations, there are a number of more specific experiments favoring the view that the dense core indeed reflects the amine levels in the vesicle. Thus, most pharmacological and experimental (electrical stimulation, denervation) results (summarized, e.g., in Hökfelt, 1968, 1970; Bloom, 1970, 1972) are in agreement with this view

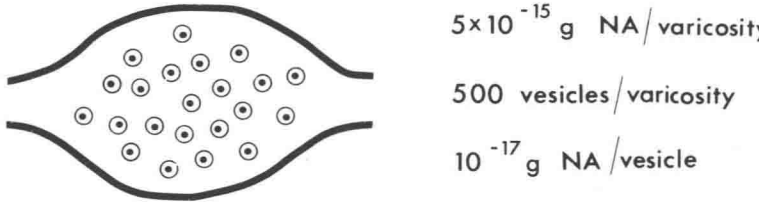


FIG. 5. Schematic illustration of an adrenergic varicosity in rat iris dilator muscle with granular vesicles (more than 95% are of the small type; see Hökfelt, 1969). The amount of NA in one vesicle can be calculated to about 10^{-17} g (see text).

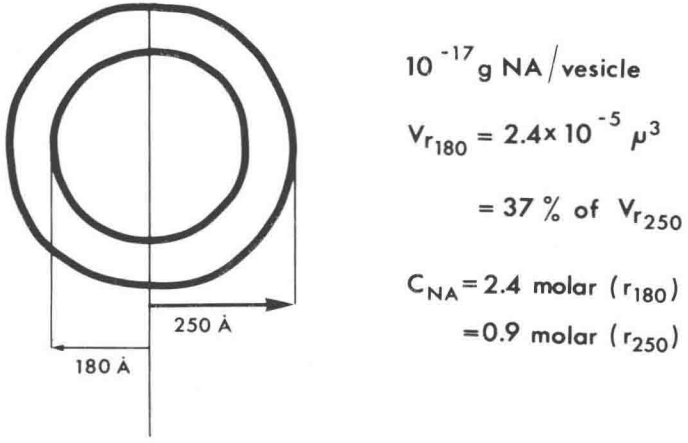


FIG. 6. Schematic illustration of a small granular vesicle with a diameter of about 500 Å and a membrane thickness of about 70 Å. The concentration of NA in the vesicle can be calculated to lie between 2.4 M (if all NA is in the interior cavity of the vesicle) and 0.9 M (if all NA is equally distributed over the whole vesicle, i.e., also within the membrane) (see text).

although some results have been interpreted to represent discrepancies (see Bloom, 1972; Goldstein and Bloom, 1970).

(5) It may, finally, be added that the term "granular vesicle" may soon give rise to serious confusion. Any successful attempt to visualize in the electron microscope a suspected transmitter substance in a synaptic vesicle will result in a "granular vesicle." Thus, the zinc-iodide-OsO_4 technique as used by Akert and colleagues (Akert and Sandri, 1968, 1970) results in electron-dense precipitates in *all* types of small synaptic vesicles and they do probably not reflect the transmitter content (Pellegrino de Iraldi and Gueudet, 1968; Matus, 1970). Furthermore, fixation with OsO_4 at +60°C reveals electron-dense precipitates in small vesicles in certain boutons, e.g., in the paraventricular nucleus. These precipitates also occur after reserpine treatment (Bloom and Aghajanian, 1968*b*) and may thus not be related to amine stores.

Although the concentrations of the amines within the vesicles are high, the absolute amount is, of course, very small, demonstrating the high sensitivity of the fixation technique required to visualize amine stores at the ultrastructural level. With the $KMnO_4$ technique, we observe dense cores in nerves in the iris dilator muscle of rats treated with a catecholamine synthesis inhibitor which lowers amine levels to about 20% of normal. With 5×10^{-3} pg NA present in one normal varicosity and 500 vesicles in this