Biochemistry and pharmacology of autonomic neurons (*)(**)

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The more we learn about the individual elements of the nervous system, their chemical nature and their physiological mechanisms, the easier it becomes to understand how drugs can influence their behavior. (LOWRY, 1963).

If the central nervous system were constituted by neurons having similar function, structure and chemical composition, it would be very difficult to justify the use of cellular analysis. We know that this is not the case and here, more than in any other organ, function is dependant on the complex coordination of different types of individual units. The smallest and simplest nucleus in the CNS is built up by different types of nerve cells. Besides nerve cells other cells are present, such as glial cells, ependymal cells, blood vessels and blood cells.

The neuron represents therefore not only the largest cell in the organism (water free weight between 5 and 50 ng. ; 1 ng = 10^{-9} g), but is also the most varied in its from, dimension and chemical composition. Terminals from other cells are connected to the cell body, the dendrites and the first part of the axon. Fig. 1 represents an isolated nerve cell body, which has been dissected out from a sympathetic ganglion and thein stained for AChE by KOELLE's method (1951).

(*) This review is based on a lecture held at the Istituto Superiore di Sanità on the 30th March, 1967.

(**) The following abbreviations have been used in the text, figures and tables :

ACh = Acetylcholine ; ^{14}CACh = ^{14}C Acetylcholine ; MeCh = Acetyl-β-methylcholine ; NE = Norepinephrine ; ^{3}HNE = tritiated norepinephrine ; 5HTP = 5-Hydroxytryptophan ; 5HT = 5-hydroxytryptamine ; DOPA = 3,4-dioxyphenylalanine ; DA = Dopamine ; GABA = γ-aminobutyric acid ; GLY = Glycine ; HI = Histamine ; PG = Prostaglandine.

AChE = Acetylcholinesterase ; BuChE = Butyrilcholinesterase ; ChAc = Cholineacetylase ; MAO = Monoamineoxidase ; COMT = Catechol-o-methyl-transferase ; DOPA-DC = DOPA-decarboxylase ; 5HTP-DC = 5HTP-decarboxylase.

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The product of the staining reaction, which is visible on the cell body, gives us an idea about the large number of connections between the cell body and terminals originating from other cells. The cell body and the dendrites

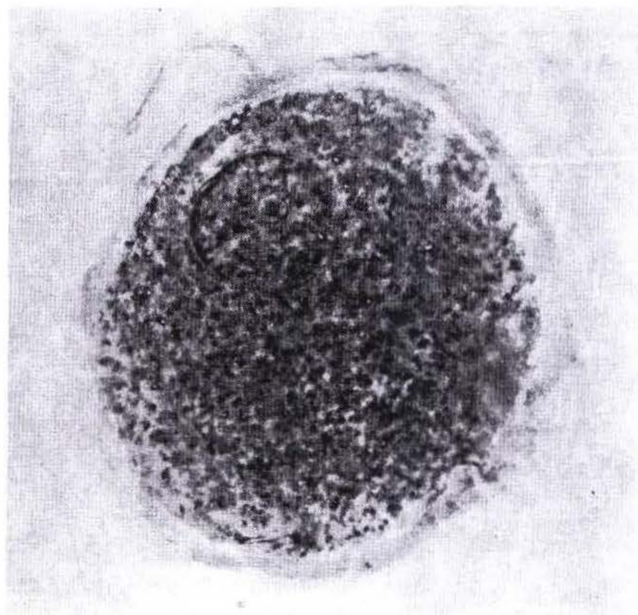


Fig. 1. — Sympathetic ganglion cell of the rat stained by KOELLE's method (1951). The AChE activity is demonstrated by small crystals covering all the cell body. (Magnification 800 ×).

are practically covered with hundreds of small structures, the synapses. The number of these synapses may be as high as 5-6.000 for cortex cells and 2-3.000 for motor neurons (CAJAL, 1909). Each neuron establishes contacts with over 1.000 neighboring cells and the electrical activity of one single fibre may influence about 5.000 neurons.

If we isolate a nerve cell by dissection under the microscope and place it in a weak solution of methylene blue it is possible to visualize thousands of small spots on each cell body, each representing a synapse.

These synapses are not at all alike morphologically, chemically or pharmacologically. Therefore, if we isolate by dissection a discrete and well defined portion of cortex, hypothalamus or mesencephalon (Fig. 2), and separate by means of centrifugal fractionation techniques the synapses present in this region from other subcellular constituents, we should still obtain a very heterogeneous material. That is, our fractions should still contain exci-

tatory, inhibitory and other types of synaptic structures (synaptosomes, Fig. 2). With further fractionation (Fig. 2) it is possible to separate the elementary particles storing the transmitter that is, the vesicles and granules. The dimension of the vesicles varies between 200 and 1.400 Å. The stored mate-

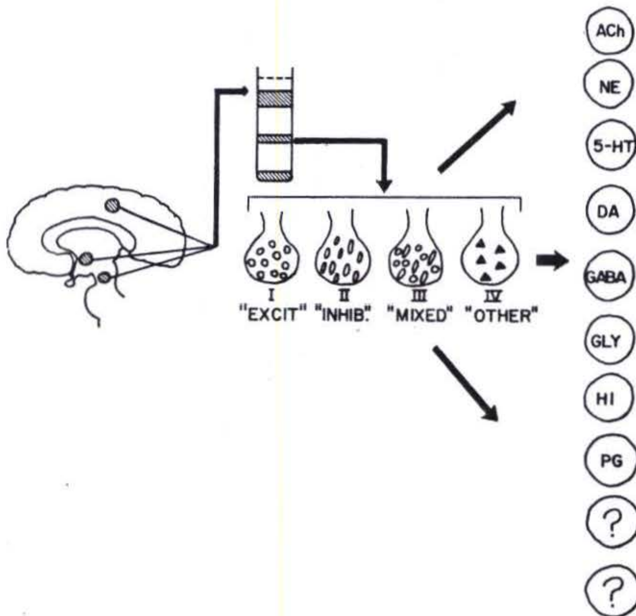


Fig. 2. — Schematic diagram of fractionation techniques for separating synapses (synaptosomes) and other subcellular structures (vesicles and granula).

rial is mainly constituted by transmitters or precursors but the presence of other substances can not be excluded. Up to the present time, stores have been found of ACh, NE and 5-HT, and it is likely that many other types will be found in the future.

The small 200 Å vesicles have been associated with ACh but, on the other hand, the same type of vesicles have been found in adrenergic synapses. This fact can not be explained at the present time. Many authors have correlated other types of larger vesicles (diameter about 500 Å, the so called « dense core ») with adrenergic terminals. This correlation seems not to be absolute and valid for all localizations in the CNS. The attempts which have been made to classify the different types of vesicles and granula are still unsatisfactory.

It has also been shown that the utilization of ^{3}HNE and the effect of many drugs acting on catecholamines differs largely in the different parts of the brain (IVERSEN, 1967).

These facts emphasize the problem of the great complexity of the CNS and illustrate the difficulty of obtaining homogeneous structures for chemical analysis. We would therefore like to stress the point that the analysis of homogenates obtained from brain or even from circumscribed regions of the brain may produce results very difficult to interprete.

THE PERIPHERAL AUTONOMIC SYSTEM AS A MODEL FOR BIOCHEMICAL AND PHARMACOLOGICAL STUDIES

In order to avoid some of the difficulties connected with the isolation of homogeneous material from the CNS we have used as a model for our investigation the different sympathetic and parasympathetic ganglia of the cat. Both physiological and pharmacological studies (SJÖQVIST, 1962 ; GIACOBINI, 1967) strongly suggested that the neurons which constitute the sympathetic ganglia have different physiological and pharmacological properties, that is, the ganglion cells may be of different natures. Histochemical studies, using staining techniques such as KOELLE's technique (1951) for AChE or the fluorescence technique for catecholamines (FALCK *et al.*, 1962) further support this view.

In the sympathetic ganglia the great majority of synapses should be, according to classical pharmacology, of the cholinergic type (Fig. 3-A). However, the postganglionic fibre can be of different types, that is, cholinergic or adrenergic (Fig. 3 B).

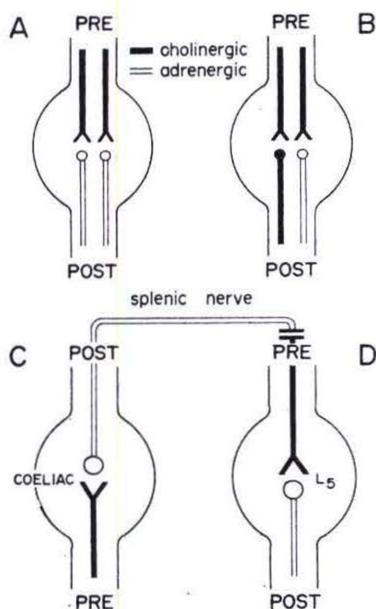


Fig. 3. — Synaptic connections in the sympathetic ganglia of the cat. A and B, synaptic connections between preganglionic cholinergic fibres and postganglionic (altern. cholinergic) adrenergic fibres. C and D, artificial anasthomosis between the splenic nerve and L5 ganglion.

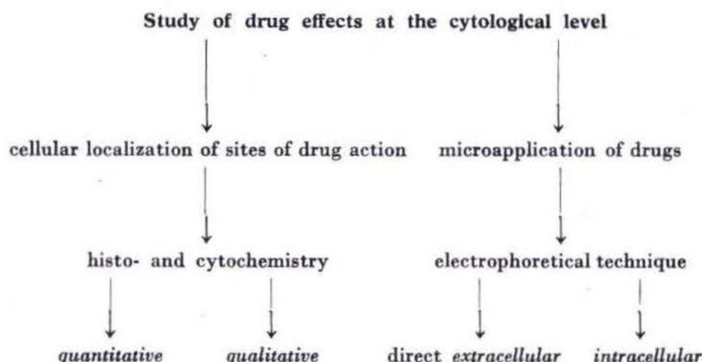
The role of ACh, as summarized by KOELLE (1962), may be two fold. First, ACh liberated at the synapse may induce the liberation of additional quanta of presynaptical ACh. Second, ACh may liberate or trigger the liberation of other transmitters from the presynaptic site. An example of functionally different postganglionic fibres is that of the cholinergic fibres originating from the L7 ganglion, which innervate the sweat glands and the vessels of the hind limbs of the cat as suggested by LANGLEY (1892).

TECHNICAL PROBLEMS CONNECTED WITH MICROCHEMICAL ANALYSIS OF SINGLE NEURONS.

If we accept the view that neuropharmacological drugs influence different parts of the CNS and different types of cells in a different way, we can understand that the use of microtechniques in the field of neuropharmacology is almost a *conditio sine qua non* rather than a technical luxury. As LOWRY pointed out (1963): « We teach that hypnotic and anaesthetic agents have a selective action on the CNS. Actually it is the other way around. It is the CNS which is selective in response to drugs ». Therefore, the more we learn about the chemical structure and function of the neurons, the easier it is to understand the mode of action of neuropharmacological drugs.

In order to study the drug effects at the cellular level we can make use of at least two different approaches: the cytochemical and the neurophysiological (Table 1). The first approach involves the microinjection of drugs,

TABLE 1.



inhibitors or transmitters directly on the neuronal membrane. The injection is obtained by means of electrophoresis with a so called multibarrelled electrode made up of several concentric micropipettes (Fig. 4). A simultaneous recording of the action potential is obtained. These experiments aim to cha-

racterize very specifically the reaction of the neuronal membrane to the administration of different types of substances, particularly those which are suspected to serve as physiological neurohumoral transmitters. The respon-

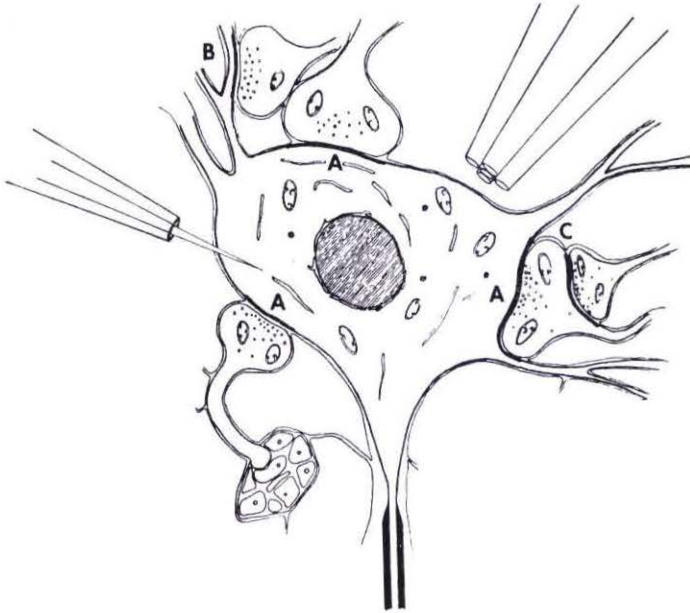


Fig. 4. — Microinjection of pharmacologically active substances or transmitters on the neuronal membrane by means of a concentric (5-barrelled) electrode. Simultaneous intracellular recording of the action potential. A, B and C represent different type of synapses (A = axosomatic, B = axodendritic, C = axoaxonal. influenced by the microiniection. The non-synaptic part of the cell membrane is occupied by glial cells. From SALMOIRAGHI & BLOOM (1964).

ses, which are recorded as a variation in the ionic conductance of the cell membrane, are related to the effect obtained after physiological or electrical stimulation of the same neuron. It should be emphasized that the strongest evidence for a substance to be identified as a « physiological transmitter » is the fact that the substance is liberated after physiological stimulation and acts on the postsynaptic membrane in a characteristic way, inducing specific conductance changes. Unfortunately, this evidence is lacking, for most CNS transmitters because of the technical difficulties inherent in such experiments.

SALMOIRAGHI & BLOOM (1964) have demonstrated that different types of neurons exist in the CNS, showing different sensitivity to various transmitters, and that the same transmitter may exert different effects upon different neurons (Fig. 5, I). For example, NE and 5HT can be both

excitatory and inhibitory for the cortical neuron (Table 2). At the same time some cells are sensitive to ACh but not to NE or 5HT. Similarly, in the nervous system of invertebrates it has been found that the activity of a single

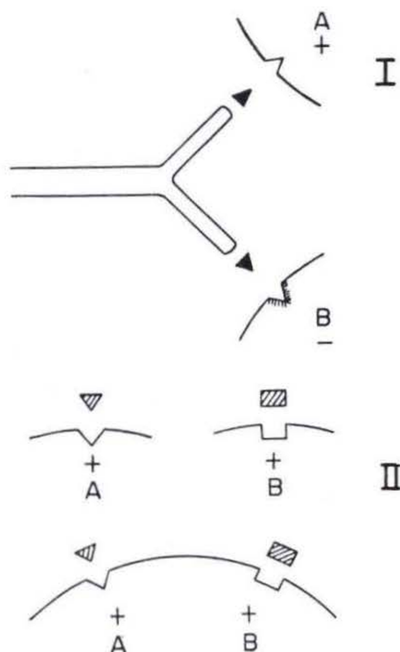


Fig. 5. — Schematic diagram of neurohumoral transmission. Triangles and squares represent two transmitters. I: To the left a presynaptic fibre; A and B represent two postsynaptic neurons. The activity of a single axon liberating the same transmitter may produce opposite effects (depolarization and hyperpolarization) upon two different postsynaptic neurons by exciting one and inhibiting the other. II: upper part: More than one transmitter, or compounds having different chemical structures, may produce a similar effect on two nerve cells (postsynaptically) and initiate the same permeability changes. Lower part: Excitatory synapses influenced by different mediators may coexist in the same neuron.

axon liberating the same transmitter may produce opposite effects upon two different postsynaptic neurons by exciting one and inhibiting the other (Fig. 5, I) (STRUMWASSER, 1962).

Furthermore, it has been found (GERSCHENFELD, ASCHER & TAUC, 1967) that more than one transmitter, or compounds having different chemical structure, may produce a similar effect on the nerve cell and initiate the same permeability changes (Fig. 5, II). Excitatory synapses under the influence of different mediators have been demonstrated to coexist in the same neuron (Fig. 5, II) (GERSCHENFELD, ASCHER & TAUC, 1967), that is, excitatory postsynaptic potentials can be produced by at least two different synaptic transmitters. In some neurons these transmitters have already been identified as ACh and biogenic amines (catecholamines and 5HT) (TAUC, 1967).

According to these results, the specificity of the synaptic response may be explained by the fact that this specificity resides in the receptor or in steps beyond the receptor, and the type of response elicited may depend

TABLE 2.

NEURON	Substances administered electrophoretically					
	ACh	NE	DA	5-HT	GLUT.	GABA
Medulla	F N D	F N D				
Pons	F N D	F N D				
Inferior colliculi	F N	N		N	F	D
Hypothalamus	F N D	F N D		F N D		
Thalamus:						
Ventrobasal complex	F		N	N D	F	D
Lateral geniculate	F	D	D	D	F	D
Caudate	F N D	F N D	F N D			
Cortex:						
Auditory, somato-sensory, sensory-motor	F N	D	D	F D	F	D
Visual	F N	D	D	F D	F	D
Cerebellum	F				F	D
Olfactory bulb	F N D	F N D		N D	F N	

Modified from SALMOIRAGHI & BLOOM (1964). F = facilitation, D = depression, N = no response.

on the characteristics of the membrane rather than of the transmitter itself (Fig. 5, I).

The final response of a neuron to a certain drug depends therefore on the combined response of different receptors, on their threshold, and on the degree of accessibility of the drug to the transmitter. In other words, because of the great complexity of synaptic organization, a certain drug having a relatively simple mechanism of action may produce a rather complicated effect by coming simultaneously into contact with inhibitory or excitatory synapses having different pharmacological « sensitivity ».

Since the neuron has developed membrane structures (receptors) having sensitivity to different types of transmitters its membrane must be pharmacologically differentiated to a high degree. This means that during development, the membrane produces different types of receptors having a specific sensitivity to different types of presynaptically liberated substances.

It is not known how the postsynaptic membrane is developed and differentiated, but it is supposed that contact with, or proximity of the presynaptic component must be of importance. The embryonic muscle, e. g., is rather uniformely « sensitive » to ACh in all its extension, but after innervation only a small and well defined region remains which maintains this sensitivity. This specific and limited region, the muscle end plate, can regress, under certain conditions, to the embryonal situation, e. g., after denervation. The neurons must therefore possess genetic information about the type of receptor molecules which will appear in its membrane. The ultimate distribution pattern and specificity of such a receptor structure is probably dependant on which type of contacts the neuron establishes with other cells during its development.

ANALYTICAL REQUIREMENTS FOR CYTOCHEMICAL STUDIES

If we now consider the second approach, that is, cytochemical analysis, we must establish certain criteria for studying the effect of drugs at the cellular level. The experiments require precise and well controlled conditions in order to avoid different types of artefacts.

These conditions are :

A) *quantitative* significance ;

B) *high specificity ;*

C) *high sensitivity* (high localization and resolution of the *local chemical effects* of a given drug action) ;

D) the presence of the drug should not interfere with the method itself ;

E) the method should be unaffected by side reactions ;

F) rapid analysis of several samples at one time ;

G) relatively low cost of chemicals and equipment.

Since the effect of a certain drug is often a consequence of its effect upon a certain enzyme or enzymes, it is important to follow the effect of the drug on the enzyme activity in restricted regions of the nervous system or, if possible, in single cells. Neuropharmacologists are also interested in studying those enzymes which take part in the metabolism of physiologically active substances like transmitters, and to specify the localization of such enzymes. We should emphasize the significance of obtaining quantitative rather than qualitative results during such a study. Most histochemical techniques (staining or fluorescence methods) have a relatively limited use in pharmacological studies because they lack quantitative significance.

The sensitivities of different types of techniques are reported in Table 3. In our laboratory we have mainly used fluorimetric, isotopic, and microma-

TABLE 3.

Sensitivity of isotope procedure and comparison to that of other methods

TECHNIQUE	SENSITIVITY (moles of measurable substrate)
Colorimetric	10^{-9} — 10^{-10}
Fluorimetric	10^{-11} — 10^{-12}
Isotopic	10^{-12} — 10^{-13}
Micromanometric (Cartesian diver)	10^{-13} — 10^{-14}

nometric techniques. The isotope techniques, which have recently been introduced in cellular analysis (BUCKLEY *et al.*, 1967*a*) have proved to be of great help. A schematic diagram of the isotopic technique developed in our laboratory for measuring different enzyme activities in single cells is shown in Fig. 6. Table 3 shows that isotopic techniques may be more sensitive than

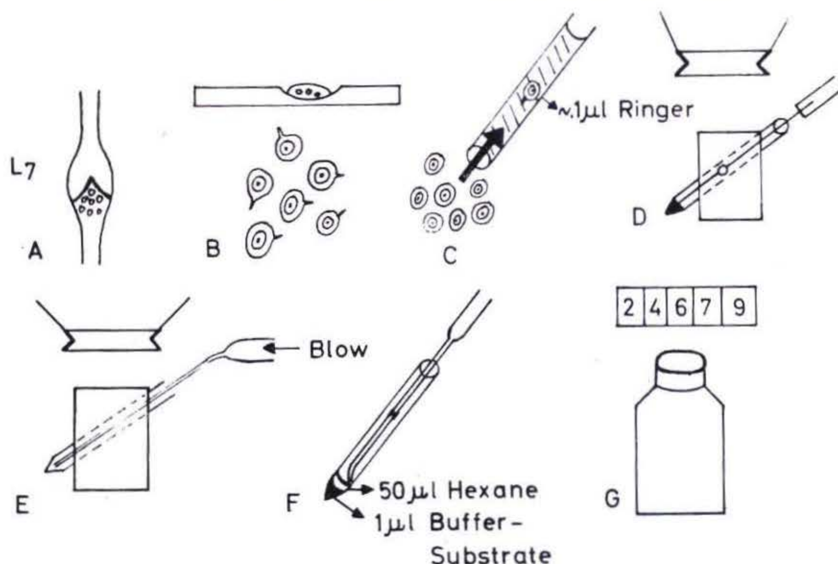


Fig. 6. — Schematic representation of the procedure for the radiochemical determination of enzyme activity (ChAc, AChE, MAO, etc.) in individual nerve cells. After removing the connective tissue capsule a small piece of ganglion is dissected out (A), and single cells are obtained from this fragment by microdissection (B). By means of a diver micropipette a single cell is washed in fresh Ringer before transferring it by the same pipette to a small tube (C and D). The small volume of Ringer carried with the cell (about 0.1 µl) is evaporated by blowing (E). The tubes containing the cells are placed in ice and 0.5-1 µl cold buffer substrate is added. To prevent evaporation, the tubes are either sealed with caps, or 50 µl hexane is pipetted onto the incubation mixture (F). After incubation and the following steps, the radioactivity is measured in a scintillation counter (G).

both colorimetric and fluorimetric techniques. On the other hand, the number of results obtained per experiment is much larger with isotopic than with micromanometric techniques.

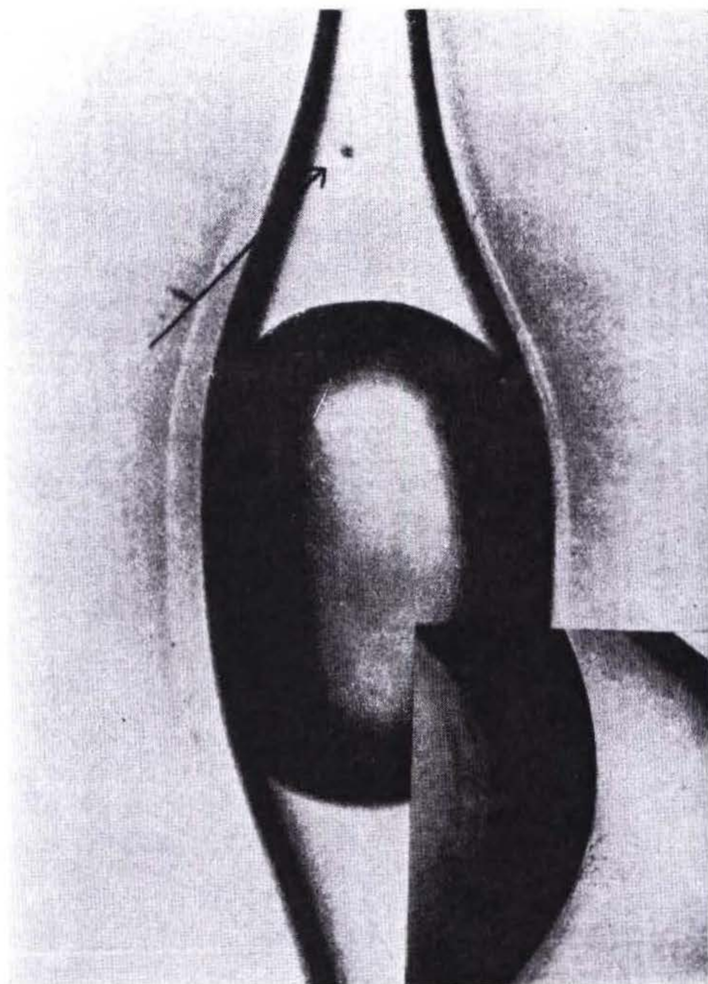


Fig. 7. — A cell body (30 μ) from a rat sympathetic ganglion is shown at two different magnifications in the diver.

Fig. 7 shows a Cartesian diver containing a single sympathetic cell body. A magnetic diver technique has recently been developed (CARLSSON & GIACOBINI, 1968) permitting an automatic recording of the variation

of the gas in the diver. The quantitative histochemical technique can be used in the study of drug action in the nervous system in the following cases:

1) Drugs which block synaptic transmission.
2) Drugs with action on the central nervous system.
3) Drugs which influence ion-transport in the nerve cell.
4) Drugs which induce changes in the nucleic acids of the nerve cell.

Different types of enzymes related to the metabolism of transmitters or precursors of the ACh or NE system have been studied by means of cytochemical techniques. Several of these techniques have been developed in our laboratory together with BUCKLEY, CONSOLO & McCAMAN (1967*a*). The concentration of NE in single cells is determined by means of the technique described by CASPERSSON, HILLARP & RITZÈN (1966). As shown in Table 4 several enzymes which are of importance for the metabolism of catecholamines like MAO, COMT, DOPA-DC etc. can now be studied in very small samples of nervous system or in single neurons. It is also possible to determine two or more different enzymes in the same neuron by combining micromanometric and isotopic methods.

TABLE 4.

Quantitative cellular assay of enzymes related to cholinergic and adrenergic transmission

Transmitter or precursor	Related enzyme	Method	References
ACh	AChE	Cartesian diver	GIACOBINI, PALMBORG & SJÖQVIST (1967).
	ChAc	Isotopic	GIACOBINI, E. & S. KOSLOW (1969).
		Isotopic	BUCKLEY *et al.* (1967 *a*; *b*).
NE	MAO	Isotopic	CONSOLO, GIACOBINI & KARJALAINEN (1968).
	COMT	Isotopic	GIACOBINI, E. & S. KERPEL-FRONIUS (1968).
DOPA . . .	DOPA-DC	Isotopic	»
5-HT	5-HTP-DC	Isotopic	»

THE CHOICE OF THE MATERIAL

As pointed out above, pharmacological and biochemical studies support the concept that different types of cell populations are present in the sympathetic ganglia. The sympathetic ganglion seems therefore to constitute a very suitable material for identifying neurons having different pharmacological and biochemical properties. Fig. 8 shows a general diagram of the analysis.

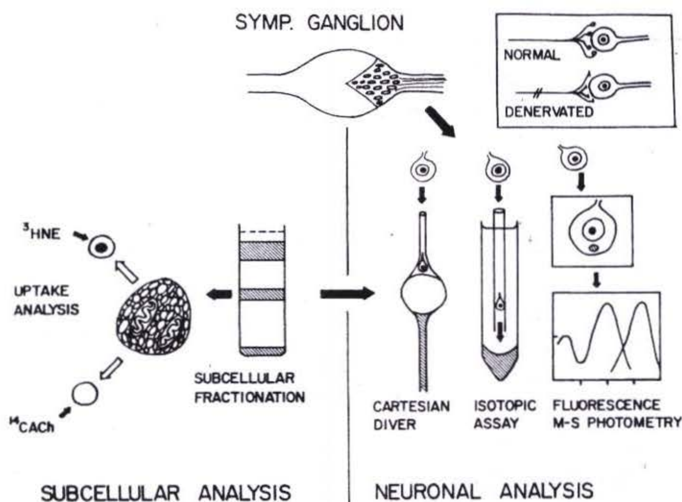


Fig. 8. — Diagram of cellular (right) and subcellular (left) analysis of enzyme activity (Cartesian diver and microisotopic methods) and monoamines (fluorescence microspectro-photometry) in isolated cells. Uptake analysis (left) for ^{3}HNE and ^{14}CACh in granules and vesicles obtained from fractions of normal and denervated sympathetic ganglia of the cat.

The levels of investigation are three : the total ganglion containing 20-25.000 neurons, single neurons isolated by microdissection and subcellular fractions obtained from the total ganglion. We feel that only the combination of these three different approaches may contribute to give a picture of such a complex material. The enzyme activity is measured first in whole ganglia rapidly isolated from the autonomic system of the cat and then homogenated. By means of homogenization, ultracentrifugal fractionation, and a sucrose density gradient it is possible to obtain different subcellular fractions (Fig. 9). Morphological controls with both light and electron microscopy are regularly performed on the ganglia as well as on the fractions.

Single autonomic neurons are dissected from the ganglia under the dissection microscope. The ganglia used for the analysis may be normal or

preganglionically denervated. This operation results in the complete destruction of the presynaptic structures (synaptic terminals) attached to the body or dendrites of the postsynaptic neuron. The isolated cells are used for three different types of cellular analysis (Fig. 8), first they may be introduced in the Cartesian diver for respiratory or enzymatic study (Fig. 7), secondly, different enzymes may be assayed using the microisotopic technique (Fig. 6), and thirdly, the level of catecholamines (noradrenaline) may be evaluated by means of fluorescence microspectrophotometry. The above steps constitute the so called « neuronal analysis ». The subcellular fractions obtained, containing synaptosomes of the different ganglia (Fig. 9), may be used for enzymatic studies or for uptake analysis.

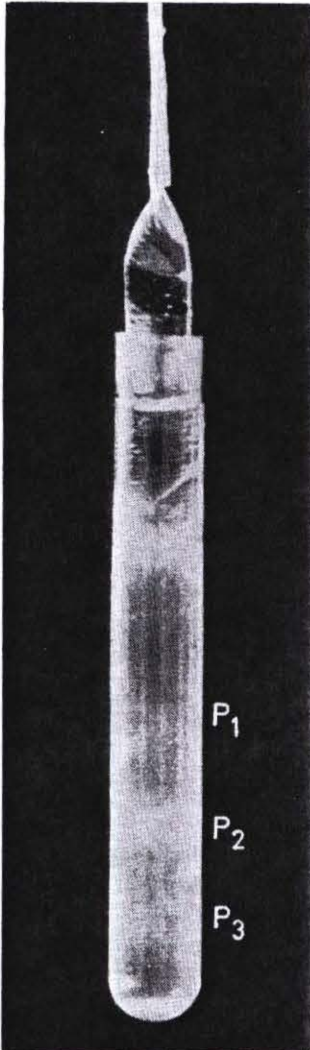


Fig. 9. — Different subcellular fractions obtained by means of ultracentrifugation and sucrose density gradient fractionation. P_1-synaptosomal layer, P_2-mitochondria + synaptosomes, P_3-mitochondrial layers. The fractions were obtained by pooling two superior cervical ganglia of the cat.

The localization of a specific enzyme in the cell body is of great importance since it has been suggested that the synthesis of transmitter substance take place in the cell body as well as in the synaptic endings. From the cell body the transmitter is transported down to the terminals through the axon. Indirect determination of the « turnover rate » for NE in different parts of the neuron of sympathetic ganglia (Costa, 1967) showed that the turnover in the cell body is significantly higher than that in the terminals.

The histochemical studies of Dahlström (1966) on peripheral autonomic neurons, showing fluorescence for catecholamines, also support the idea that the storage particles for noradrenaline are synthesized in the cell body and then transferred through the axons to the terminals.

Among the questions which can be answered by the types of analysis mentioned above are the following :

— are « adrenergic » neurons capable of metabolizing ACh as well ?
— are « cholinergic » neurons capable of metabolizing NE ?

The compatibility of the occurrence of AChE in various types of neurons with the fact that these neurons are presumably non-cholinergic has been a matter of discussion. Histochemical results have also suggested the presence of AChE in adrenergic neurons or fibres. This fact has been brought forward to support the hypothesis of BURN & RAND (1962) which involves ACh in the adrenergic transmission mechanism.

THE DISTRIBUTION OF FOUR TRANSMITTER ENZYMES IN AUTONOMIC GANGLIA

When comparing the relative activity of AChE and ChAc in some autonomic ganglia of the cat (Fig. 10 and 11) it became evident that a close correlation exists between ChAc and AChE activities. Some ganglia, for example

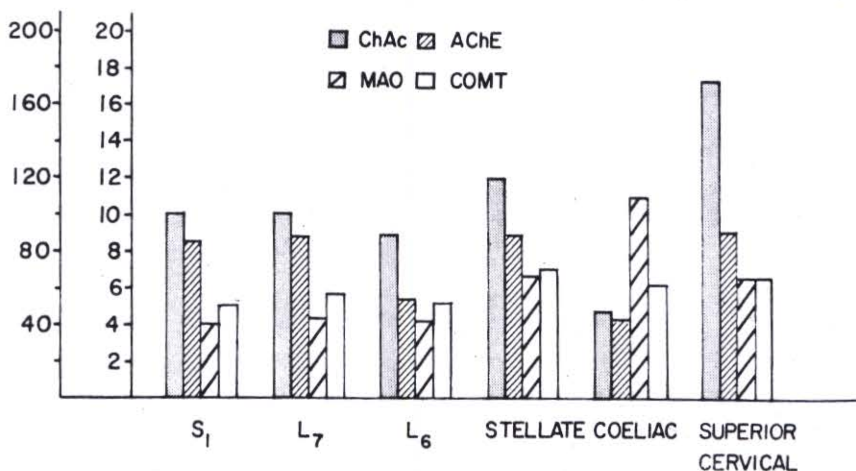


Fig. 10. — The enzyme activity (ChAc, AChE, MAO and COMT) of four enzymes in the sympathetic ganglia of the cat.

ChAc activity is expressed in moles ACh $\times$ 10^{-13}/hour/μg wet. (Scale 0-200). AChE activity is expressed in μmoles HAc $\times$ 10^{-3}/min./mg. (Scale 0-20). MAO activity is expressed in moles of product $\times$ 10^{-12}/hour/μg wet. (Scale 0-20). COMT activity is expressed in μmoles of product/hour/mg wet. (Scale 0-20).

stellate and cervical superior, show particularly high activity. The ciliary ganglion shows the highest activity of all the autonomic ganglia investigated (Fig. 11).

With respect to their ChAc and AChE activity the ganglia can be ranked in the following progressive order : coeliac, L6, Sl, L7, stellate, superior cervical and ciliary. The lowest level of ChAc and AChE activity is found in the

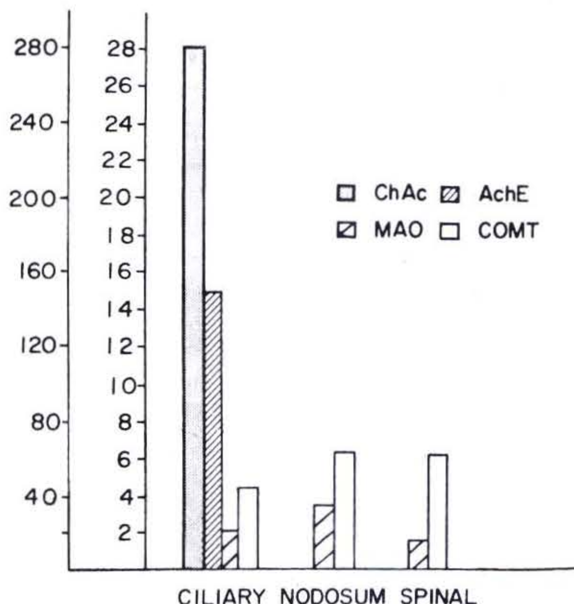


Fig. 11. — Distribution of enzyme activity (ChAc, AChE, MAO, and COMT) in parasympathetic and sensitive ganglia of the cat. The units used are indicated in Fig. 10.

coeliac ganglion which also exhibits the highest MAO activity. Inversely, the ciliary ganglion which shows the highest ChAc and AChE activity, has the lowest MAO activity (about half of L7).

Nodose and spinal ganglia show rather low but appreciable MAO acticity (GIACOBINI & KERPEL-FRONIUS, 1968). Coeliac and inferior mesenteric, which show the highest MAO activity of all ganglia, have the most abundant system of adrenergic synaptic terminals of all sympathetic ganglia. On the contrary, the ciliary ganglion contains only a few synaptic adrenergic structures (HAMBERGER, NORBERG & UNGERSTEDT, 1965) and has the lowest MAO activity.

L6, L7 and S1 show a remarkably similar MAO activity which correlates well with the reported values of the NE content for these ganglia. (CONSOLO, GIACOBINI & KARJALAINEN, 1968).

THE EFFECT OF PREGANGLIONIC DENERVATION

Preganglionic denervation results in a loss of both ChAc and AChE activity in the ganglia (BUCKLEY *et al.*, 1967*b*) (Fig. 12). The decrease in the enzyme activity is about 98 % in all ganglia for ChAc, but only 58 and 64 % for AChE, indicating that the former enzyme is more specifically located at the presynaptic level (Fig. 12). The data presented in Fig. 12 show that in the

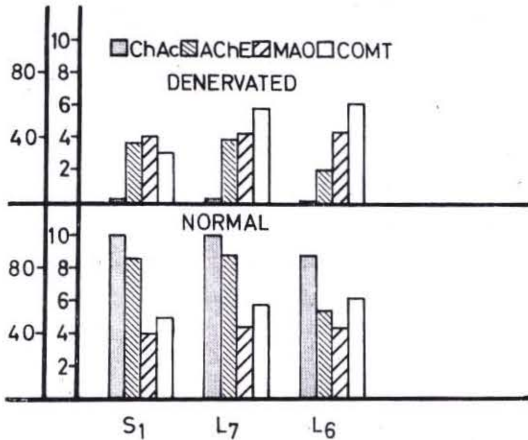


Fig. 12. — The effect of denervation on four enzymes (ChAc, AChE, MAO and COMT) in three different sympathetic ganglia of the cat. The units used are indicated in Fig. 10.

presynaptically denervated S1, L7 and L6 ganglia neither the MAO nor the COMT activity are changed.

Both presynaptic fibres and synaptic boutons are completely degenerated one week after denervation (HUNT & NELSON, 1965 ; GRILLO, 1966).

Clumping and agglutination of synaptic vesicles has been observed within 6 hours of the operation. The synaptic vesicles show a tendency to move away from the synaptic site, whilst intraaxonal cytolysomes appear. The axonal cytolysomes appear to be expelled into the Schwann cell's plasma (Szentagothai, personal communication, 1968). The latter changes are apparent within 12 to 18 hours after the operation.

In the investigation of GIACOBINI, PALMBORG & SJÖQVIST (1967) on the L7 ganglion of the cat it was shown that here, as in the rat ganglia (GIACOBINI, 1957), the total AChE activity of normal cells shows a wide variation (about 50 fold). The cells with low activity represent the majority of the population, whilst neurons with very high activity are rare (Fig. 13 and 14). Only one of the 40 cells investigated lacked measurable activity (Fig. 13). This study also showed a characteristic distribution pattern of

enzyme activity in the L7 ganglion of the cat (GIACOBINI, PALMBORG & SJÖQVIST, 1967).

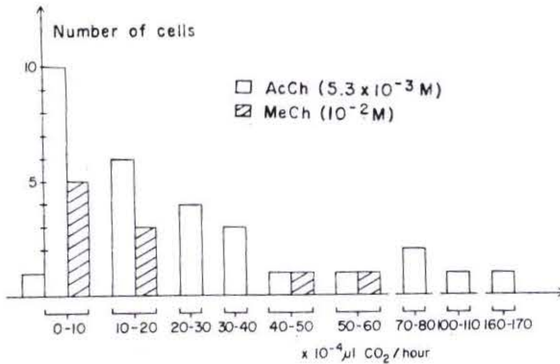


Fig. 13. — Frequency distribution of ChE activity (in µl of $CO_2 \times 10^{-4}$/hour) in individual sympathetic cell bodies from the normal L7 ganglia of the cat.

After denervation, there was a remarkable decrease in average enzyme activity as measured with both substrates (MeCh and ACh) (Fig. 14). As

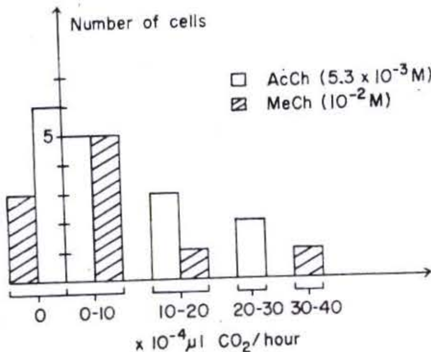


Fig. 14. — Same distribution as in Fig. 13, but from denervated ganglia.

much as 35 % of the denervated cell population lacked measurable enzyme activity. In cell bodies with measurable activity the values varied more than 10 fold. This study demonstrates the presence and the high variability of enzyme activity in the postsynaptic pericarial enzyme.

According to histochemical studies (FREDRIKSSON & SJÖQVIST, 1962) preganglionic denervation causes the disappearance of AChE from presynaptic terminals but does not affect the AChE of the cell bodies. Innervated cells were found to exhibit a considerably higher AChE activity than denervated ones (Fig. 14). Both ChE (BuChe and AChE) are present in the ganglion cells of the cat but BuChE is confined to glial cells (KOELLE, 1951; GIACOBINI, 1959) (Fig. 15).

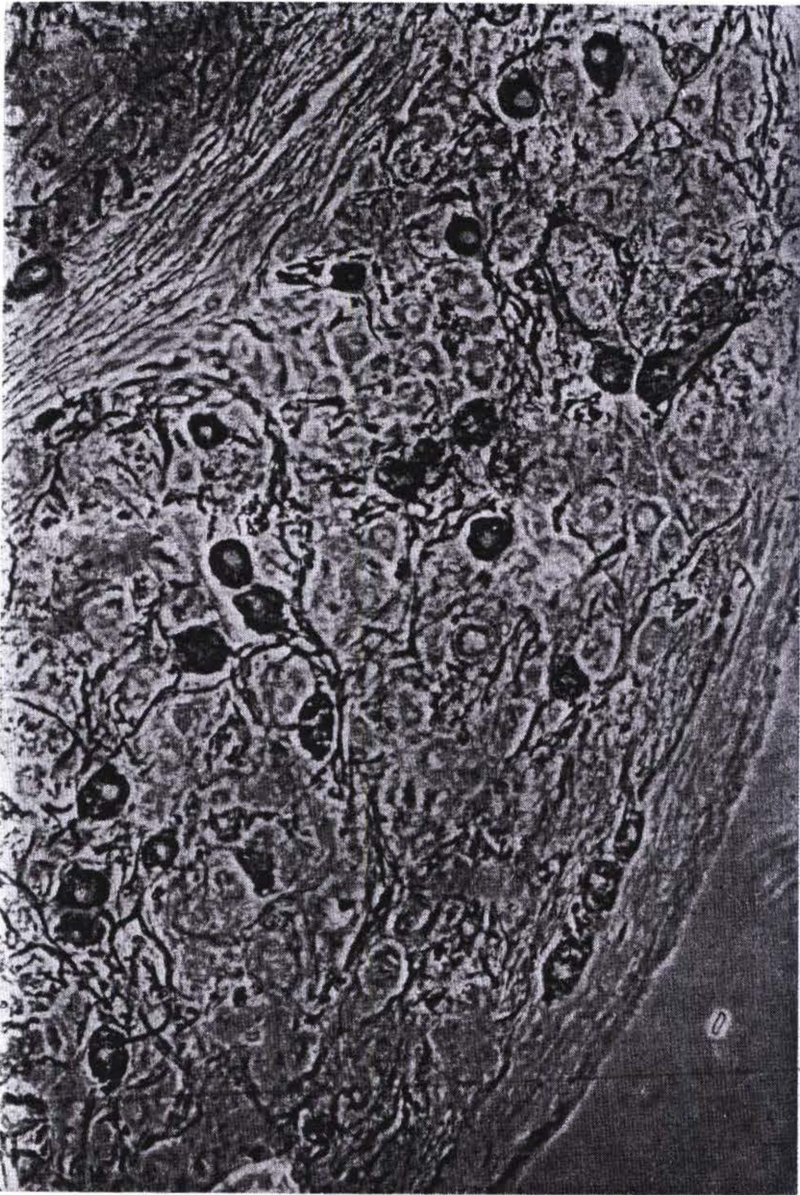


Fig. 15. — L7 ganglion of the cat stained for AChE with Koelle's method (1951). Cryostate section. Magnification about 150. Normal ganglion with AChE in a few cell bodies and in surrounding fibres. Some cells are more heavily stained than others.

ChAc activity was measured in 405 isolated cell bodies from the L7 ganglion (BUCKLEY *et al.*, 1967*b*). 216 cells were dissected from 7 normal ganglia and 189 from 3 denervated ganglia. The 27 normal cells with measurable ChAc show activities from 13.6 to 0.8 $\times$ 10^{-13} moles ACh/hour (Fig. 16). These cells represent 12.5 % of the investigated population. Cell bodies with low ChAc activity represent the majority of the population, whilst those with very high activity are rare. After denervation the proportion of active cells remained approximately the same (13.2 %) (Fig. 16). The difference between the

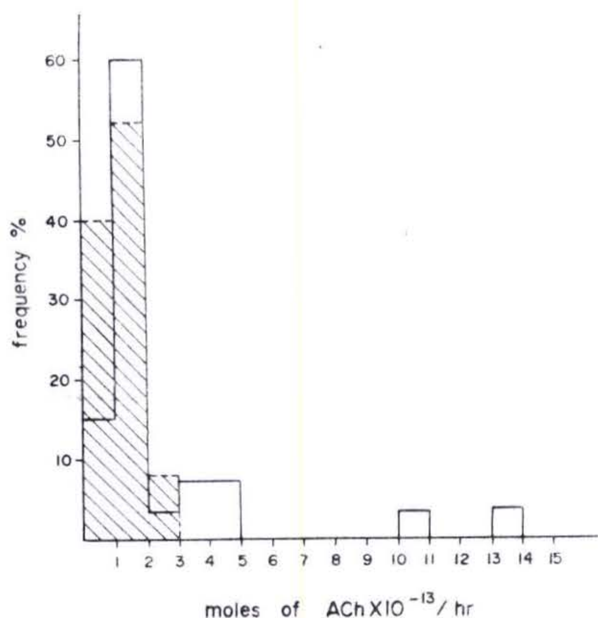


Fig. 16. — Frequency distribution of ChAc activity (in moles of ACh $\times$ 10^{-13}/hour) in individual sympathetic cell bodies from the L7 ganglia of the cat. The shaded areas represent the frequency distribution after denervation.

individual values becomes less after denervation and a decrease in the average enzyme activity from 2.6 to 1.25 $\times$ 10^{-13} moles ACh/hour occurs as well.

5 experiments were performed determining MAO activity on 242 isolated cell bodies from the L7 ganglion (CONSOLO, GIACOBINI & KARJALAINEN, 1968). 142 cells were dissected from three control ganglia and 100 from the two ganglia 32 days after denervation. 103 cells of the control ganglia (Fig. 17) showed measurable MAO activities between 1 and 42 $\times$ 10^{-12} moles of product/hour. These represented 73 % of the cells investigated and their mean activity was 5.5 $\pm$ 0.57 $\times$ 10^{-12} moles of product/hour. The majority of the population were cell bodies with low MAO activity while a few had a very high activity, above 20 $\times$ 10^{-12} moles of product/hour (Fig. 17). Of the denervated ganglia 69 cells (Fig. 17) showed measurable MAO activities between 2 and 46 $\times$ 10^{-12} moles of product/hour. This represented 69 % of the cells investigated and their mean activity was 6.5 $\pm$ 1.0.

The majority of the population was constituted by cell bodies with activity below 28×10^{-12} moles of product/hour. The proportion of active cells in each experiment varied in the normal ganglia between 67.5 and 78.5 %

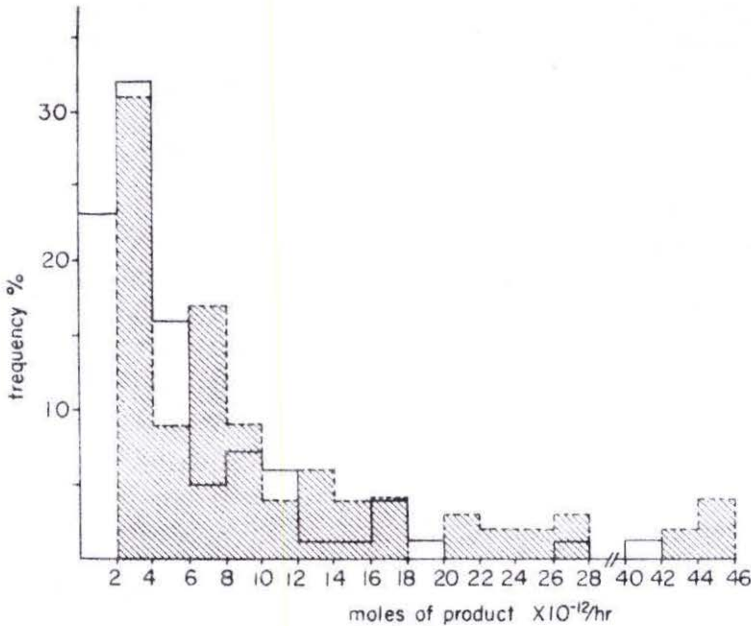


Fig. 17. — Frequency distribution of MAO activity in moles of product $\times$ 10^{-12}/hour in normal and denervated sympathetic ganglion cells of the L7 ganglion of the cat. The shaded areas represent the distribution after denervation.

and in the denervated ganglia between 69 and 70 %. A significant increase in the average enzyme activity after denervation from 5.5 ± 0.57 to $6.5 \pm 1.0 \times 10^{-12}$ moles of product/hour was found.

The fact that the level of MAO activity in the whole ganglion is unchanged (Fig. 12) despite a significant increase in the level in the single cells indicates that the MAO activity of the presynaptic fibres and their terminals, and of the non-nervous (glial) component of the denervated ganglion is probably decreased. MAO activity has been found (Axelrod, personal communication, 1968) to be five times higher in the synaptosomal than in other fractions, which may explain this decrease. This is accompanied by a concomitant increase in the MAO activity of the postsynaptic component (cell body). Since MAO activity is higher in the synaptic region than in other parts of the nervous tissue, the degeneration of this region would be expected to produce a marked decrease of the total activity of the ganglion.

An increase in norepinephrine content of the postsynaptic cell bodies of sympathetic ganglion cells (FISCHER & SNYDER, 1967) as well as of cen-

tral monoaminergic cells (DAHLSTRÖM & FUXE, 1965) as a result of both pre- and postsynaptic denervation has been reported.

These findings suggest that at least two biochemical alterations (NE and MAO) in postsynaptic cell bodies occur after presynaptic denervation. To test whether the increased MAO activity is due to a proliferation or hypertrophy of mitochondria, the activity of other specific mitochondrial enzymes in the same material is now under investigation.

It should be emphasized once more that HUNT & NELSON (1965) and GRILLO (1966) found complete degeneration of the synapses but no obvious morphological changes in the postsynaptic cell bodies of denervated autonomic ganglia from the frog and the rat. The few synapses which may resist degeneration in the rat ganglia (GRILLO, 1966) are thought to arise from small neurons, the « interneurons » of WILLIAMS (1967).

Our experiments (CONSOLO, GIACOBINI & KARJALAINEN, 1968) with isolated cell bodies show that more than 70 % of the cells in L7 contain the enzyme required for the oxidation of the monoamines. These figures agree relatively well with the percentage of cells (about 85 %) in which monoamine fluorescence can be demonstrated (HAMBERGER, NORBERG & SJÖQVIST, 1963). Our results also suggest that in L7 the cells storing monoamines may be identical with those containing MAO, and MAO may be more specifically localized than was previously considered on the basis of histochemical studies (KOELLE & VALK, 1954). MAO would appear to occur mainly in monoamine containing neurons (Fig. 18).

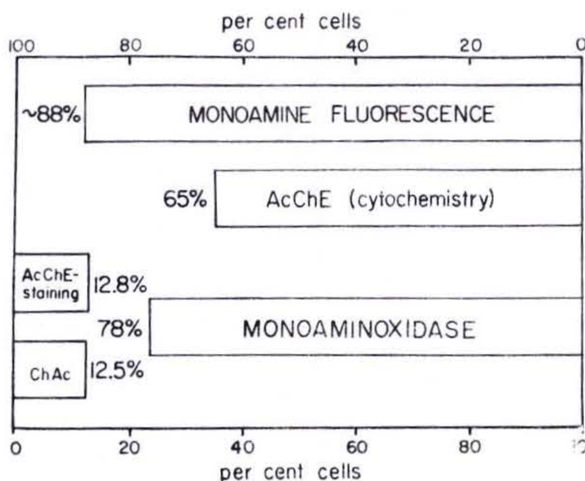


Fig. 18. — Diagram of the percentage of the cells from L7 ganglion of the cat containing AChE, ChAc, monoamines and MAO. The scheme shows that the number of ganglion cells, which contain monoamines, MAO and AChE activity (right) considerably exceeds the proportion of cells lacknig monoamines but containing ChAc or showing heavy staining for AChE (left).

The lack of correlation between COMT activity and either MAO activity or catecholamine level in the ganglia found by GIACOBINI & KERPEL-

Fronius (1968) suggests that the enzyme may not be selectively present in the « adrenergic » cells. This result is in agreement with the view that COMT may act outside the neuron itself but close to the receptor site in the synapse.

COMPARISON OF ENZYME ACTIVITY IN SYMPATHETIC, PARASYMPATHETIC AND SENSITIVE GANGLIA

Both AChE and ChAc show considerably higher activity in the ciliary ganglion than in sympathetic ganglia. In fact the highest values of the two enzymes were found in the ciliary and superior cervical ganglia, and the lowest in the coeliac ganglion. The coeliac ganglion exhibits the highest MAO activity, inversely the ciliary ganglion shows the lowest MAO activity, that is, about one half of L7.

Nodose and thoracic spinal ganglia show rather low MAO and COMT activity (Giacobini & Kerpel-Fronius, 1968). Coeliac and inferior mesenteric show the highest MAO activity of all sympathetic ganglia. The ciliary ganglion contains only a few synaptic adrenergic structures (Hamberger, Norberg & Ungerstedt, 1965) and has the lowest MAO activity.

The ChAc activity in the dorsal spinal roots of the cat is very low, in the order of 0.02 mg ACh/g dried tissue/hour, as compared to 10 mg/g dried tissue/hour in the ventral roots (Hebb, 1962).

The AChE present in the dorsal spinal roots of the cat is also very low (3-13 μM MeCh/g/hour) as compared to that of the sympathetic system (cervical sympathetic nerve, 440 μM MeCh/g/hour) (Hebb & Krnjevic, 1962). The ChAc and AChE levels agree quite well with the ACh content of these regions (Hebb & Krnjevic, 1962).

CHOLINERGIC AND ADRENERGIC CELLS IN THE SYMPATHETIC GANGLIA

Our results support the idea that the sympathetic ganglia of the cat contain two distinct cell populations: firstly a « cholinergic » population representing in L7 about 10-15 % of the ganglion cells (Fig. 18). These cells are characterized by the presence of ChAc, high concentrations of AChE and the absence of monoamine fluorescence and MAO activity. Secondly, an « adrenergic » population comprising about 73-88 % of the ganglion cells, which exhibits fluorescence for NE and MAO activity. It contains low or moderate AChE and no measurable ChAc activity. The first population is indicated in the left part and the second in the right part of Fig. 18. The

distribution pattern of the two populations in L7 ganglion of the cat is shown in Fig. 19.

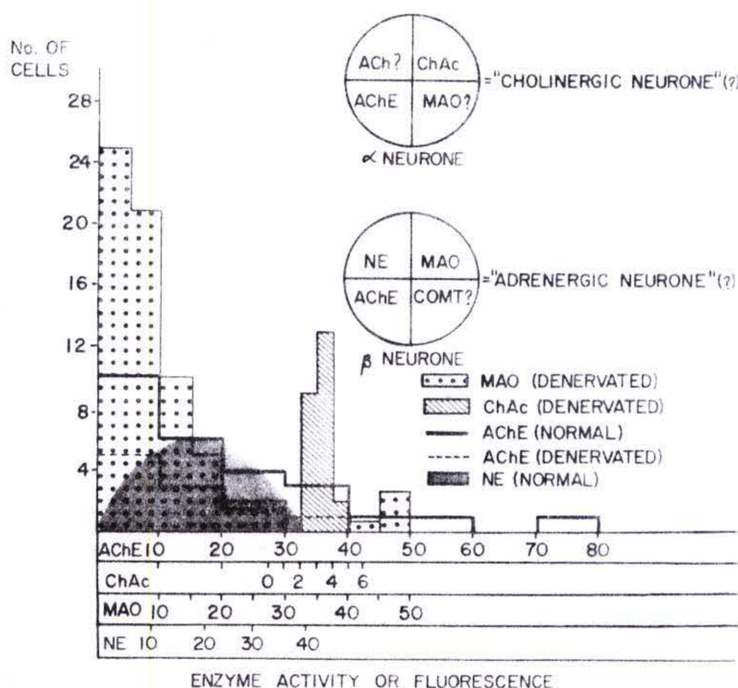


Fig. 19. — Distribution patterns of AChE, ChAc, MAO and NE, in the L7 ganglion of the cat. The enzyme activity and the degree of fluorescence for monoamines is expressed in arbitrary units. See text.

GENERAL COMMENTS

Neurophysiologists have provided, by means of their elegant studies, useful and solid hypotheses about synaptic transmission mechanisms, and they have also described the different phases of excitation and inhibition in the nerve cells.

These studies have been possible only because of the introduction of the intracellular recording technique, and with its help it has been possible to study the response of single neuronal units. If such a powerful tool as the microelectrode had not been discovered, our knowledge about the physiology of the nervous system would still be very vague and fragmentary.

It is by no means unlikely that neurochemists and neuropharmacologists in a relatively short time will be able to describe with equal precision the

pharmacological and chemical features of differents types of neurons including « adrenergic » and « cholinergic » neurons.

The results so far obtained in our laboratory may already help us to answer a few questions which are of fundamental significance when trying to understand the mechanism of action of drugs acting on the autonomic nervous system.

In summary :

in the sympathetic ganglia of the cat the levels of AChE and ChAc are rather well correlated when looking at the ganglion *in toto*, however, the AChE containing neurons are much more numerous than the ChAc containing neurons.

ChAc is localized mostly preganglionically in the nerve terminals whereas AChE is localized both pre- and postganglionically. The postganglionically localized AChE is present not only in the postsynaptic membrane but also in the cell body and in the postganglionic fibres. These enzymes are also highly concentrated in the postsynaptic terminals, a structure which is unfortunately not readily available for chemical analysis.

AChE has therefore a more « diffuse » distribution than ChAc. Actually AChE is present also in « adrenergic » neurons while ChAc is probably restricted to « cholinergic » neurons (Fig. 19). Therefore, the presence of AChE in the neuron does not represent a sure indication that the neuron is « cholinergic ».

CONCLUSIONS

By using a simplified model of a functional unit of the nervous system such as a sympathetic ganglion, we have tried to throw some light on problems connected with synaptic transmission, e.g. :

— is it possible for a neuron to metabolize different types of transmitters ?

— do true « adrenergic » or « cholinergic » neurons exist as is thought in classical pharmacology ? The answer to this question seems to be negative and, in our opinion, a neuron may be « less or more » « adrenergic » or « cholinergic ». An example of this is the presence of AChE in neurons showing high concentrations of catecholamines.

In this connection we should not forget, as pointed out earlier, that the same neuron may be stimulated or inhibited by a single transmitter, or that the same transmitter may have different physiological effects upon different cell types. In other words, the autonomic neuron is probably equipped with the tools for participating in several transmitter lines. The exact physiological significance and the potential of the neuron in this respect is still completely obscure. However, it is possible that the neuron possesses a biochemical me-

chanism for synthesizing and inactivating transmitters which may be modified according to the physiological demand.

The next question is :

— is it possible that a certain transmitter such as ACh may be present in a neuron which is « non-cholinergic » or, more exactly, is it possible that several types of transmitters may be present in the same neuron ? At the present moment we have no evidence for or against this hypothesis. However, this possibility can not be ruled out.

Has the cell body the capacity of synthesizing transmitters ?

Our results clearly indicate that ChAc is present and concentrated in certain neurons and therefore the pericarial synthesizing machinery can probably be activated whenever the transmitter is needed.

Has the cell body enzymes for the inactivation of the transmitter ? AChE is present in a large percentage of sympathetic cells, and MAO is equally present in many cell bodies. However, we have so far no direct evidence for the presence of COMT in cell bodies.

Of interest is the fact that the transmitters are metabolized and synthesized at least in two different parts of the neuron, that is, in the cell body and in the synapse. This supports the theory that ACh and catecholamines are formed in the cell body and then transferred through the axon to the terminals. We know, however, that the axon (KOENIG, 1967) may also be able to synthesize enzymes involved in transmitter metabolism, e.g., AChE.

The picture of the autonomic neuron emerging from our data is one of a cell having latent biochemical potential. It has the capacity to synthesize or inactivate the transmitter not only near or close to the place where the transmitter is used (synapse) but also far from it, that is, in the axon or in the cell body.

The extraneuronal (glial) localization of transmitters or related enzymes is still a matter of discussion. Work is in progress in our laboratory in order to clarify this point.

The enzymatic pattern of the autonomic neuron is rather complex and consists of several components : therefore, the presence in a neuron of a specific enzyme or even of the transmitter itself can not be taken as a conclusive demonstration that this neuron is « adrenergic » or « cholinergic ».

The last question is :

— how versatile is the neuron regarding the synaptic transmission mechanism. Has the adult neuron the possibility of changing or modifying the transmission mechanism necessary for its function ? The data reported above could indirectly indicate that a possibility for dynamic changes in the neuron exist.

Fig. 3 A and B show the two possible physiological synaptic connections present in a sympathetic ganglion. In A two preganglionic cholinergic fibres impinge on two « adrenergic » neurons, in B one of the fibres impinges on a « cholinergic » and the other on an « adrenergic » neuron. In the first case there is a switch at the synaptic side from a « cholinergic » to an « adrenergic » metabolism. In both types of junctions ACh is acting physiologically.

In order to examine the possibility of changing or modifying these connections which have been established during development, it should only be necessary to exchange the type of presynaptic nerve ending. In a model which is presently being investigated (GIACOBINI & KERPEL-FRONIUS, 1968) we have tried to modify these connections by making an anasthomosis between a « pure » adrenergic nerve (preganglionically) and an adrenergic cell. In this case the synapse which is acting physiologically under the influence of ACh is now challenged to perform its activity with another transmitter (noradrenaline). At the present stage we can not yet say whether this experiment has been successful, however, there are some indications that the plasticity of the synapse may be far more pronounced than was earlier believed.

The implication of such a versatility in other processes such as learning mechanisms, behavior and memory is obvious, however, there is still much more to be learned before we understand the dynamic characteristics of the neuron.

Summary. — New quantitative microchemical techniques for the measurement of enzyme activity, metabolites and transmitters in single nerve cells have become available recently. Several enzymes related to the metabolism of transmitters can now be studied with high precision and accuracy in individual neurons.

The cell population in the L7 ganglion of the cat has been used as a model to study the correlation between chemistry and pharmacology of autonomic neurons. Both cholinergic fibres (sweat secretor and vasodilator) and adrenergic fibres (vasoconstrictor) originate from this ganglion. The activity of several enzymes (AChE, ChAc, MAO and COMT) as well as monoamines have been studied at three different levels, total ganglion, single cells and subcellular fractions.

The results obtained by these analyses show that the sympathetic ganglia of the cat contain two distinct cell populations ; firstly, a « cholinergic » population, representing in L7 about 10-15 % of the ganglion cells. This population is characterized by the presence of ChAc, high concentrations of AChE and the absence of monoamine fluorescence and MAO activity. The second is an « adrenergic » population, about 72-88 % of the ganglion cells. It exhibits fluorescence for NE and MAO activity and contains low or moderate AChE activity and no measurable ChAc activity.

In the sympathetic ganglia of the cat the levels of AChE and ChAc are rather well correlated when looking at the ganglion *in toto*. However, the AChE containing neurons are much more numerous than the ChAc containing neurons. ChAc is localized mostly preganglionically in the nerve terminals whereas AChE is localized both pre- and postganglionically. The postganglionically localized AChE is present not only in the postsynaptic membrane but also in the cell body and in the postganglionic fibres These enzymes are also highly concentrated in the postsynaptic terminals. AChE has therefore a more « diffuse » distribution that ChAc. Actually AChE is present also in « adrenergic » neurons while ChAc is probably restricted to « cholinergic » neurons. Therefore the presence of AChE in a neuron does not represent an indication that the neuron is « cholinergic ».

A model is presented for examining the possibility of changing or modifying physiological synaptic connections in the autonomic nervous system.

The implication of synaptic plasticity in learning, behavior and memory mechanisms is emphasized.

Riassunto. (*Biochimica e farmacologia del neurone del sistema autonomo*). — Nuovi metodi microchimici quantitativi, adatti alla misurazione di attività enzimatica in singole cellule nervose, sono stati recentemente introdotti.

Diversi tipi di enzimi partecipanti al metabolis modi sostanze trasmettitrici del sistema nervoso (acetilcolina, noradrenalina, ecc.) possono ora essere studiati con grande precisione in neuroni isolati.

La popolazione cellulare del settimo ganglio lombare del gatto venne usata come modello onde studiare alcune correlazioni chimiche e farmacologiche nel neurone del sistema autonomo.

Da questo ganglio prendono origine sia fibre colinergiche (per le ghiandole sudoripare e vasodilatatrici) che fibre adrenergiche (vasocostrittrici). L'attività di diversi enzimi quali : acetilcolinesterasi, colinoacetilasi, monoaminoossidasi e catecol-o-metiltrasferasi e la concentrazione di monoamine (noradrenalina e adrenalina) vennero studiate a tre diversi livelli ; nel ganglio totale, nelle singole cellule e in frazioni subcellulari.

I risultati di tale analisi dimostrano che la popolazione dei gangli simpatici del gatto è costituita da due distinti gruppi cellulari. Il primo è un gruppo « colinergico », rappresentante in L7 circa il 10-15 % delle cellule gangliari. Questa popolazione è caratterizzata dalla presenza di colinoacetilasi, da alte concentrazioni di acetilcolinesterasi e dell'assenza di fluorescenza per le monoamine e assenza di attività monoaminoossidasica. Il secondo gruppo è costituito da una popolazione di cellule « adrenergiche » che costituisce circa il 72-88 % delle cellule gangliari. Tale popolazione consta di cellule che dimostrano fluorescenza per la noradrenalina ed attività monoaminoossi-

dasica, e che contengono bassa o moderata attività acetilcolinesterasica ma non colinoacetilasica.

Esaminando il ganglio *in toto*, i gangli simpatici del gatto dimostrano una buona correlazione tra i valori dell'acetilcolinesterasi e quelli della colinoacetilasi. A livello cellulare, tuttavia, si osserva che i neuroni aventi attività acetilcolinesterasica sono assai più numerosi di quelli aventi attività colinoacetilasica. La colinoacetilasi è localizzata prevalentemente nella parte pregangliare e nelle terminali sinaptiche, mentre l'acetilcolinesterasi è localizzata sia nella parte pre- che in quella postgangliare. L'acetilcolinesterasi localizzata al livello postgangliare è presente non solo nella membrana postsinaptica ma anche nel corpo cellulare e nelle fibre postgangliari. Entrambi gli enzimi sono altamente concentrati nelle terminazioni postgangliari.

Si può dire perciò che l'acetilcolinesterasi dimostra una distribuzione meno specifica e più diffusa che la colinoacetilasi. Di fatto l'acetilcolinesterasi è presente anche in neuroni « adrenergici » mentre la colinoacetilasi è specificamente concentrata nei neuroni « colinergici ». La presenza di acetilcolinesterasi in una cellula nervosa non può essere ritenuta dunque come una indicazione che il neurone è « colinergico ».

Per esaminare la possibilità di scambiare e modificare le connessioni sinaptiche del sistema autonomo dell'adulto, funzionanti fisiologicamente, si può usare un modello presentato dall'autore mediante il quale si reinnerva un ganglio simpatico con fibre « adrenergiche » pure.

L'importanza di conoscere il grado di plasticità e rimodellamento a livello della sinapsi viene discussa e messa in relazione a problemi quali quelli dell'apprendimento, del comportamento e dei meccanismi della memoria.

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