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## Chemical Transmission in Invertebrate Central Nervous Systems and Neuromuscular Junctions

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## I. INTRODUCTION

This review attempts to survey the chemical mechanisms involved in synaptic transmission in the central nervous system and the neuromuscular junctions of invertebrates. However, nerve junctions with cardiac and visceral muscles are not considered here since they have been recently reviewed elsewhere (162, 590, 738).

It does not seem necessary to emphasize the interest of the subject to those physiologists who are well aware that an important part of our knowledge of the basic mechanisms of nerve function has been obtained in invertebrate preparations. For the more "anthropocentric" physiologists, who may still believe that only studies in vertebrate or even in mammalian species are germane to a knowledge of the structure and function of human beings, this is a reminder that to date the molecules involved in chemical transmission and the mechanism of their action on membranes are the same both in vertebrates and invertebrates.

The study of transmitters in the invertebrate nervous systems presents some

advantages, especially in the use of certain microtechniques on individual neurons that are difficult to perform in vertebrates. In many invertebrate phyla the size of the neurons facilitates both intracellular potential recording and microapplication of drugs. In almost all the invertebrate nervous systems it is possible to set up *in vitro* preparations and thus control the environment of neurons. Moreover it has recently become possible to identify, isolate, and submit to microchemical analysis individual neurons (327, 698).

Despite these particular advantages, however, the work on transmitters is hindered by some important limitations. The first important shortcoming is that, in spite of the wide acceptance of chemical mediation as the main mechanism of communication between vertebrate or invertebrate neurons, there are few cases where a given transmitter substance has been proven beyond reasonable doubt to be linked to the function of a given synapse. The crucial demonstration of such a relationship consists fundamentally of proving that at the arrival of an action potential at the presynaptic ending the substance is released in necessary and sufficient quantity to produce the physiological effect observed. This proof has not yet been attained for any synapse. Strong evidence approaching this desideratum has been obtained for ACh<sup>1</sup> in the neuromuscular junction of vertebrates (see 460), for NE in some peripheral adrenergic synapses (see 430), and for GABA in the inhibitory neuromuscular junction of crustaceans (see 512). In other cases neurobiologists appeal to a more or less eclectic list of criteria that a substance is expected to fulfill to be accepted as a transmitter in a given synapse (204, 264, 312, 686, 924). In this way the need for rigorous evidence is replaced by an accumulation of indirect proofs (morphological, biochemical, pharmacological, physiological) that are expected to reach the point when a certain "general consensus" accepts the substance as a likely transmitter. As a result it is probably easy to discard a transmitter candidate, but it is very difficult to attain the conviction that a compound actually has a transmitter role. A Japanese proverb says that "one may even worship the head of a sardine if one really wants to believe." The abundant literature on invertebrate chemical synapses shows that the credibility of many authors on the transmitter role of a substance relies only on their personal faith. This reviewer has tried to set the level of his exigence as high as possible in judging transmitter candidates, but some readers may possibly find him worshiping a sardine.

A more particular shortcoming of transmitter studies on invertebrates is that from the enormous amount of extant species of invertebrates only a limited number in each phylum has been used in the experiments on synaptic transmission. There-

<sup>1</sup> Abbreviations used in this paper: ACh, acetylcholine; AChE, acetylcholinesterase; CA, catecholamines; DA, dopamine; DOPA, dehydroxyphenylalanine; dTC, *d*-tubocurarine; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; HMT, hexamethonium; 5-HT: 5-hydroxytryptamine, serotonin; 5-HTP, 5-hydroxytryptophan; MAO, monoamine oxidase; NE, norepinephrine; TEA, tetraethylammonium; TTX, tetrodotoxin; EPSP, IPSP, excitatory or inhibitory synaptic potential; EJP, IJP, excitatory or inhibitory junctional potential; mEJP, mIJP, miniature EJP or IJP;  $E_{EPSP}$ ,  $E_{IPSP}$ ,  $E_{EJP}$ ,  $E_{IJP}$ , reversal potentials of synaptic and junctional potentials;  $E_{drug}$ : reversal potential of drug response;  $E_{ion}$ , equilibrium potential of ionic species;  $[ion]_o$ , external concentration of a ionic species;  $[ion]_i$ , internal concentration of an ionic species;  $E_m$ , membrane potential.

fore, since it is difficult to resist the temptation of generalizing the results, these generalizations must be taken very cautiously.

Studies on chemical transmission in some lower phyla having a nerve net (coelenterates) or whole classes of some higher phyla (onychophorans or arachnids) are not treated because of the scarcity of data about them. It is more interesting to analyze only those phyla where the available information may be significant. In addition an effort has been made to avoid references to isolated and/or meaningless observations.

In the last decade both partial and comprehensive reviews on invertebrate synapses have been published (162, 267, 268, 738, 825), and it has been difficult to avoid some overlap with them. Some reviews have covered aspects of synaptic function in invertebrates related with nervous integration, plasticity, and behavior (452, 453, 825); none of these subjects is treated here.

## II. NEMATODES

### A. Neuromuscular Junctions

Nematodes present an interesting feature known since Schneider's work in 1860 (see 178): the muscle cells are prolonged by a series of branches, these branches approaching the nervous system to establish synaptic contacts. The main studies in neuromuscular junctions have been done on *Ascaris lumbricoides*.

1) *Structural background.* *Ascaris lumbricoides* presents a tegument formed by a thick epidermis, covering a dense hypodermis. All the space between these layers and the digestive system of the animal is almost filled by somatic muscle cells (see details in 178, 731, 732). Therefore, the round transversal section of the animal is almost filled by muscle. There are two small nerve cords running ventrally and dorsally along the body.

It is rather difficult to realize the complexity of the muscle cells in aniline-stained sections. Recent work in morphology and electron microscopy (36, 178, 385, 730-732) has clarified different aspects of muscle cell morphology, particularly the relationships of muscle cells with each other and with nerve cell axons, in both *Ascaris lumbricoides* and *Parascaris equinum* (known before as *Ascaris megalcephala*).

The somatic muscle cell consists of a rather huge cell body containing the nucleus and an important amount of glycogen that becomes depleted by starvation (730, 732). The muscle cell bodies, called bellies, prolong themselves in two other processes of the cell: *a*) lamelliform prolongations, called spindles, that run longitudinally along the body under the hypodermis, forming a palisade-like structure; *b*) thin prolongations, called arms, that emerge from the bellies and converge over both neural cords, where they intermingle and form a complex syncytial arrangement. The electron-microscope study has detected two types of junction (732) at the level of this syncytial structure. One type corresponds to five-layered junctions between muscle arms, which Rosenbluth (732) described with features known today

to correspond to "gap" junctions. The others take place between muscle arms and axons. The nerve cords are well surrounded by the dense hypodermis, which shows gaps or holes at some special spots around the cord. Across these gaps an apposition between axonal branches and muscular arms is observed. The regions of membrane apposition show an extracellular gap of 300–400 Å and at this level the axonal membrane shows a particular density. Near the inner face of this density synaptic vesicles of 300 Å appear clumped and a cluster of giant dense mitochondria is usually present. These regions correspond to neuromuscular junctions *en passant* (36, 730–732).

2) *Junctional transmission.* Jarman (438) first recorded with intracellular microelectrodes from the bellies of *Ascaris* muscle cells resting potentials of around –30 mv. Some cells discharged spikelike signals 2–10 mv in amplitude.

The electrophysiology of the *Ascaris* muscle and neuromuscular junctions was thoroughly analyzed by Del Castillo and his colleagues (178, 179, 182–186, 191). They found (179, 186) resting potentials varying between –30 and –40 mv according to the distance from the belly where the microelectrodes were implanted to the syncytium. The cells discharge action potentials (amplitude up to 40 mv) that originate at the syncytium region (179, 184, 186). The rhythmic discharge of these spikes is myogenic, and although influenced by nerve activity they may be discharged in absence of any nerve influence (186).

When the nerve cord is stimulated electrically inhibitory and excitatory effects on muscle cells may appear either mixed or in isolation (184, 186, 335). When recorded with intracellular micropipettes the excitatory effects consist of depolarizations accompanied by an acceleration of spike discharge frequency. Inhibitory effects may be obtained more frequently by nerve stimulation and consist of transient hyperpolarizations of variable duration with inhibition of the spike discharge (186). The amplitude of these hyperpolarizations also depends on the position of the impaled belly with respect to the syncytium region: i.e. the longitude of the arm relating both cell regions (184, 186). No precise measures of a reversal potential of these hyperpolarizations are available. In the best recording conditions the peak of a maximal hyperpolarization reached –46 mv (see below). In some cases repeated stimulation of the cord was followed by the "spontaneous" discharge of IJP's, probably due to repetitive firing of inhibitory fibers (186).

In some cells showing widespread spike activity, a low-amplitude synaptic noise composed of small depolarizing and hyperpolarizing transients may be observed (186). When recording near the syncytium the discrete noise components become larger. This noise is probably of synaptic origin and may correspond to mEJP and mIJP. Due to the recording conditions it was not possible to demonstrate whether it actually corresponds to a spontaneous release of transmitter or to postsynaptic potentials resulting from the spontaneous discharge either of interneurons or of nerve fibers (186).

3) *Excitation and acetylcholine.* Acetylcholine was found in whole-animal extracts of *Litmosomoides carinii* and in *Ascaris* heads and body walls (601). *Ascaris* heads (where a large part of the nervous system is located) contain 15 times more ACh than the body walls (0.39 µg/g of wet tissue against 0.025 µg/g). In *Litmosomoides* it could be proved that the substance was ACh itself (601).

The existence and the localization of cholinesterase in nematodes have been more controversial. Baldwin and Moyle (44) found that effects of ACh on *Ascaris* muscle did not fall off with time and suggested that no cholinesterase was present, but Bueding (97) detected biochemically the enzyme in *Litmosomoides carinii* and in much smaller quantities in *Ascaris* whole body. Moreover, anticholinesterases like eserine potentiated the responses of *Ascaris* muscles to ACh (650). Histochemical methods also suggest that cholinesterases are present in the muscle bellies, the muscle arms, and at the level of the syncytium (556). Electron-microscope histochemistry demonstrates the presence of acetylcholinesterase at the level of neuromuscular junctions of *Parascaris equinum* (36).

ACh causes contraction of *Ascaris* muscles at concentrations down to  $10^{-6}$  g/ml (44, 650). Atropine does not affect this action (44), but nicotine, succinylcholine, and decamethonium are effective in evoking *Ascaris* muscle contraction (650, 839). Del Castillo and his colleagues (182) analyzed the effects of ACh on *Ascaris* muscle using microelectrophysiological methods. ACh perfusion depolarizes the muscle fibers and reduces the amplitude of the muscle action potential. This depolarizing effect depends both on the ACh concentration and on the initial value of the potential. For instance, in a cell showing a potential of -32 mv, ACh  $3.3 \times 10^{-6}$  w/v may cause a potential decrease of 16 mv (182). Local iontophoretic application of ACh to the muscle cell membrane does not reveal any ACh sensitivity at the level of the belly, the spindles, or the region of the arms closer to the belly. On the contrary, ACh receptors appear mainly concentrated in the syncytium region, especially near the nerve cord region (182). dTC blocks the effect of ACh either applied by perfusion or iontophoresis (182, 650); dTC by itself does not change the resting potential or the spike discharge. The anticholinesterase agents eserine (650) and neostigmine (182) prolong and potentiate the effects of ACh. Moreover neostigmine exerts agonistic effects by inducing depolarization of the muscle cells and increase of the spike frequency (182). These effects have been attributed to a possible potentiation by the anticholinesterase agents of the action of ACh spontaneously released from neuromuscular junctions (182). This hypothesis is attractive but needs further confirmation. Unfortunately there is no information available on the effects of cholinergic drugs on junctional potentials, either spontaneous or evoked by electrical stimulation.

It is not yet possible to decide whether ACh has an excitatory transmitter role to the muscle of *Ascaris* or other nematodes, but the experimental evidence suggests that such may be the case.

*4) Inhibition and GABA.* An interesting light on the problem of muscle inhibition in *Ascaris* has been shed by observations in anthelmintic therapeutics. Some 15 years ago it was established that piperazine is an effective anthelmintic that paralyzes *Ascaris* (782, 794). The stimulating action of ACh on *Ascaris* muscle is blocked by piperazine, although the contractions produced by electrical stimulation of the muscle are not affected by piperazine. Therefore it was claimed that piperazine affects nematode neuromuscular junctions in a curare-like way (335, 650).

Intracellular recording shows that concentrations varying from  $10^{-5}$  to  $10^{-2}$

g/ml cause hyperpolarization accompanied by inhibition of the spike firing (184). The amount of hyperpolarization depends both on the concentration of the drug and on the position of the impaled muscle belly with respect to the syncytium region, the effect appearing to be most intense when recorded at arms near the nerve cords (189). This means that the site of action of piperazine, like ACh, is probably at the syncytium region. This finding has been also confirmed by iontophoretic application of piperazine to different regions of the cell (184).

When piperazine is perfused at the same time that inhibition of muscle is elicited by nerve cord stimulation (see above), piperazine potentiates the effects of nerve-evoked inhibition, even in cases where its effects on the membrane potential consist only of a very slight hyperpolarization without alteration of the spike firing (183).

The changes produced by piperazine are then very similar to those caused by inhibitory nerve stimulation. When a part of the extracellular concentration of  $\text{Cl}^-$  is replaced by  $\text{SO}_4^{2-}$ , the hyperpolarizing effects of piperazine are diminished (183). It is then possible to conclude from the summarized data that piperazine does not act on *Ascaris* muscle like a cholinergic blocking agent but more likely as an agonist of the inhibitory transmitter, possibly on the same receptors sites and causing a similar change of conductance as the transmitter (183).

GABA was found to exert a powerful hyperpolarizing action on *Ascaris* muscle cells (185). At low concentrations ( $10^{-7}$  M) GABA exerts a small but significant depolarizing action on the muscle cells. When GABA concentration is increased further the muscle cells hyperpolarize and the spike firing is abolished (185). The hyperpolarization attains a maximal amplitude of 15 mv when GABA is perfused at concentrations of  $10^{-5}$  M (185). This maximal hyperpolarization level is similar to the level reached when piperazine is applied (183), and this value also coincides with the peak of the inhibitory junctional potentials evoked by nerve stimulation (186). This potential level of about -47 mv probably corresponds to the  $\text{Cl}^-$  equilibrium potential, because it is equal to the membrane potential of a muscle after being soaked in a  $\text{Na}^+$ -free solution, when the membrane potential is thought to be under the exclusive control of  $\text{Cl}^-$  ions (184).

No further data are available on GABA action and no attempts have been made to try the effect of known blocking agents to GABA receptors on IJP's evoked by nerve stimulation or on the subthreshold noise. Further biochemical and physiological work is necessary to determine if GABA plays some role in the inhibition to *Ascaris* muscle fibers.

### III. ANNELIDS

#### A. Central Nervous System

Information on the transmitters acting at annelid central synapses is rather scarce, but growing knowledge about the function of identified neurons and their connections inside the central nervous system (CNS) and at the periphery allows

us to foresee in the very near future precise evidence linking known transmitters to specific synapses.

1) *Structural background.* Detailed accounts of the anatomy of the CNS of annelids may be found in several books (105, 363, 555, 575).

Since the features of the CNS of different types of annelids are extremely similar, some characteristics of the CNS of leeches and earthworms, the annelids most studied from the point of view of neurophysiology, are described.

The CNS of the leech consists of 34 ganglia: 13 of them fuse to form the brain and two other ganglionic masses, one subpharyngeal and another anal; the other 21 ganglia form the ventral nerve cord. Nerve roots emerge at both sides of the ganglia connecting them to the periphery. The ganglia are joined by nerve connectives.

In earthworms and other oligochetes a system of giant fibers formed by segmented giant axons runs along the entire length of the nerve cord (137; see 645). They arise from nerve cells located in each ganglion and their diameters, according to the species, may vary from 10 to 200  $\mu\text{m}$ . In the earthworm the segmented axons conduct impulses in both directions like a continuous-core conductor (99, 215, 736). The septa between the segments show areas where the two segmental membranes almost fuse and the extracellular space disappears, as in gap junctions (140, 356). Transmission at this level is bidirectional, of the electrotonic type (457). The axons may have collateral connections, which in the earthworm are also interconnected by electrotonic junctions (940). However, in some sabbellid worms they are chemically operating synapses (349).

Each leech ganglion is formed by around 350 monopolar neurons grouped in packets by a low number of voluminous glial cells (10, 365, 712, 744, 745; see 143). These ganglia show bilateral symmetry and are avascular. The ganglia of the earthworm are vascularized and generally the neurons are smaller and the glial cells much more numerous than in the leech (10, 107; for recent papers see 140, 558).

In the leech the packet unipolar neurons are enveloped by a glial cell. The unipolar neurons send their axons to form a complex neuropile in the core of the ganglion. The fine structure of the neurons and the glia has been analyzed in *Hirudo* and *Theromyzon* (143, 338, 343). The nerve cells are surrounded by a highly developed trophospongium formed by glial cell prolongations and the lacunar spaces separating them. Glial cells also send infoldings that penetrate deeply into corresponding invaginations of the neurons. Some neurons show several kinds of granulated and agranulated vesicles in their perikarya. Some of the granulated vesicles resemble in size "elementary neurosecretory granules"; others are smaller and very osmophilic, resembling "adrenergic granulated vesicles" (see below). In the neuropile, synapses may be identified by the presence of thickenings in regions of contact between axons with microvesicles attached to one of the sides. The synaptic vesicles in the endings of the leech neuropile may be electron-lucent vesicles of 200–250 Å that sometimes may appear mixed with "neurosecretory" granules (143). Synapses with the same morphological appearance may be found in the neuropile of the earthworm ganglia, where they appear to contain different classes of microvesicles: "empty" small vesicles of 200–250 Å (140, 192), dense-core

granulated vesicles of 600 Å, and large neurosecretory granules (140). The clear vesicles may appear mixed with one or the other type of granulated vesicles. The same vesicles may appear both in leeches and earthworms at the perikarya and along axons (140, 143, 343, 659, 683). A surprising and unexplained finding in the earthworm is the presence of a number of tiny axonal branches making typical synaptic contacts with the giant axons of the ventral cord (140).

2) *Properties of neurons.* Leech neurons are easily impaled *in situ* or after opening the glial packets. They may show resting potentials of -30 to -60 mv. Some of the neuron somata, like those of the so-called Retzius cells (see below), the Leydig neuron, and the sensory neurons (see below), may fire overshooting action potentials of 60-70 mv (216, 348, 641). Other neuronal somata are not actively invaded by the spike and their action potentials do not show an overshoot, their amplitudes never reaching more than 20-30 mv (641).

The action potential recorded in the Retzius neurons depends on the external  $\text{Na}^+$  concentration, being blocked in a  $\text{Na}^+$ -free medium (306). The spike undershoots are due to an increased  $\text{K}^+$  permeability (48, 50).

There are no detailed studies about the ionic regulation of the membrane potential of the leech neurons. However, in some sensory cells an electrogenic sodium pump may be activated by tetanic stimulation. The neurons may be activated either antidromically by stimulating their receptive fields or directly through an intracellular microelectrode (49, 642). Repetitive spike discharges thus caused are followed by a posttetanic hyperpolarization that may be abolished irreversibly by ouabain (49, 642). A reversible decrease of the amplitude of the hyperpolarization may be observed when the preparation is perfused with strophanthidin or when the neurons are cooled (49, 642). Since this posttetanic hyperpolarization is not affected when suitable changes in both  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  external concentrations completely block synaptic transmission in the ganglion, a synaptic origin of the hyperpolarization may be discarded. During the posttetanic hyperpolarization the neuronal membrane becomes very sensitive to  $\text{K}^+$  changes in the environment, and this may have an impact on the integrative functions of the neuron since repetitive firing of a neighboring neuron or group of neurons may lead to an increased  $\text{K}^+$  concentration in the extracellular clefts (49, 50, 642, 643). This is well demonstrated by the decrease in amplitude of the spike undershoots (cf. 281) or by measuring the glial membrane potential (50) since the glial cells membranes of the leech behave as a potassium electrode (530, 643; see 531).

3) *Identification of neurons and connections.* The bilateral symmetry of the segmental ganglia and the uniformity of cell components from one ganglion to another along the nerve cord have facilitated the histological recognition of some neurons such as the "colossal" cells of Retzius or the so-called Leydig neurons (712).

We owe to Nicholls and his colleagues Baylor, Stuart, Purves, and Odurih (48, 51, 52, 641, 644, 790, 791) a remarkable work of anatomical and physiological identification of neurons and of many connections between neurons and with the periphery. They have identified 7 pairs of central sensory cells, 14 pairs of excitatory motoneurons, and 3 pairs of inhibitory motoneurons present in each segment of the ganglionic chain, except the ganglia at both ends.

For identifying the neurons both histological and electrophysiological methods were used, particularly the intracellular injection of the fluorescent dye Procion yellow (786). The criteria used to identify the sensory neurons were morphological (shape, size, position, pattern of branching) and physiological (action potential discharge, response to skin stimulation, etc.). On each side of the ganglion seven sensory cells may be observed (48, 641): *a*) three "touch" cells that respond with a high-frequency burst of overshooting spikes to a light touch in the skin of the corresponding ipsilateral segment; *b*) two "pressure" cells that discharge a low-frequency burst of spikes when pressure is exerted on the skin; and *c*) two other "noxious" sensory neurons also responding with low-frequency discharge to more violent stimuli like pinching, squeezing, or cutting the skin of the corresponding segment (48, 641). The two latter types of neurons show a very slow adaptation and the noxious cells may still fire after removal of the stimulus (641). Whether these cells are primary sensory neurons is not yet clear, since there may be another receptor neuron at the periphery, but it is evident that they are sensory since their axons run to (or from) the periphery and the abolition of synaptic transmission inside the ganglion by increasing  $Mg^{++}$  and removing  $Ca^{++}$  from the environment does not affect the discharges produced in these neurons by skin stimulation (48, 641). The sensory receptive fields of these neurons are discrete and show constant size from segment to segment (641).

Many of the ganglion motoneurons (28 excitatory and 6 inhibitory) have been identified by Stuart (790, 791). Procion yellow injections and recordings of antidromic axon firing demonstrate that the axons of these neurons travel in the ganglion roots. The direct stimulation of each neuron is followed by the immediate recording of a single action potential in one of the roots and either an excitatory or an inhibitory junctional potential in one of the segmental muscles. These effects of stimulation of the neuron were not altered by changes in  $Mg^{++}$  and  $Ca^{++}$  in a compartment containing the ganglion, causing complete abolition of the synaptic transmission into the ganglion. In this condition the activation of muscle located in a separate compartment did not occur through a second neuron (790, 791). A given motoneuron or inhibitory interneuron innervates the same territory from segment to segment. In the same segments the innervated territories are arranged in a sort of "quilt pattern" (790, 791).

The sensory neurons are connected through synapses located at the neuropile (51, 52). An action potential evoked by either cutaneous or intracellular stimulation in any one of the touch cells causes a postsynaptic potential in the other five touch neurons located on both sides of the same ganglion and the activation of the three ipsilateral touch cells of both adjacent ganglia (51). The synaptic activity thus evoked is complex and comprises a short-latency coupling depolarizing potential followed with some delay (2–4 msec) by EPSP's and IPSP's. The appearance of the latter is irregular and shows a variable latency. The coupling potential between the touch cells is due to the activation of an electrical synapse that is very particular: it rectifies in such a way that depolarizing currents may pass through it bidirectionally, but not the hyperpolarizing pulses (51). This synapse differs from the other rectifying synapses known in other nervous systems (37, 294, 644), but resembles well the functions described between retinular neurons of *Limulus* (86).

Changes in  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -suppressing synaptic transmission in the ganglion do not affect this coupling potential (51). The EPSP's and the IPSP's recorded after the coupling potential are probably chemical, especially the IPSP that may be reversed by intracellular injection of  $\text{Cl}^-$  (51).

The IPSP's in all the ipsilateral touch neurons are produced by the recurrent activation of the same interneuron, since the inhibitory potentials appear simultaneously in the three touch cells of the same side. They do not seem to be synchronous with the IPSP's recorded in the contralateral touch cells (51). Such mechanisms seem to ensure some integration between neurons both innervating the same segment and providing innervation to adjacent ipsilateral hemisegments (51). The discharge of spikes in pressure neurons may produce inhibitory synaptic potentials in the touch neurons, which then appear synchronically in the ipsilateral neurons. Pressure-neuron discharge also evokes EPSP's in the noxious neurons. The activation of touch neurons does not affect the neurons responding to other sensory modalities (51).

The monosynaptic connections of the three types of sensory neurons with two large identified motoneurons have been thoroughly analyzed (644). The discharge of an action potential in any one of the sensory neurons evokes a peculiar synaptic activity in these large motoneurons. These motoneurons are located in the same half-ganglion as the sensory neurons but their axons decussate and thus innervate muscle fibers located in the contralateral half-segment (644). A spike in the touch cells produces in these motoneurons a short-latency EPSP through the activation of a rectifying electrical synapse of the classical type, i.e. rectifying synapses of crayfish and fish brain (37, 294), a depolarization passing only from sensory cell to motoneuron but not in the opposite direction (644). The activation of noxious cells evokes in the motoneuron monosynaptic EPSP's of probably chemical origin (2-4 msec in latency), since high- $\text{Mg}^{++}$  and low- $\text{Ca}^{++}$  media abolish them. The pressure cells connect with both motoneurons through a double mechanism: the discharge of a pressure-cell spike evokes an electrically mediated EPSP followed by a chemical EPSP (644). All these connections allow any one of the 14 sensory neurons to evoke a contraction of the contralateral longitudinal segmental muscle (644).

Continuation of this work will probably bring out more information on the reflex pathways and on the synaptic mechanisms in the leech CNS. Hopefully transmitter work will follow the same pathway, since as seen in the following section only very fragmented pieces of evidence are available on the possible transmitters involved in all the mechanisms described by Nicholls and his colleagues.

4) *Acetylcholine*. Evidence of a cholinergic excitatory innervation of leech and earthworm body muscles is presented in section II B. Very probably the motoneurons of these animals are cholinergic, but we do not yet possess any direct biochemical evidence on this point.

The only pharmacological observation on precise effects of ACh on annelid CNS is that it depolarizes and excites the Retzius neurons (502). Nicotinic agonists mimick this action well, especially carbachol (948). Muscarinic agonists in lower concentrations may cause inhibition of the Retzius cells, but excite them when applied in higher concentrations (948). Hexamethonium, decamethonium, benzoquinonium, and atropine may antagonize the effects of ACh (502).

5) *Monoamines.* Chromaffin cells were first described in the central ganglia of leeches by Poll and Sommer (697) at the beginning of the century. Further work repeatedly demonstrated that six cells in each ganglion, among them the Retzius neurons, give a positive chromaffin reaction (300, 684, 891). The idea that these neurons may contain epinephrine comes from the claim by Biedl (71) and Gaskell (301) that this catecholamine could be assayed in ganglia extracts. Vialli (891) challenged this view, pointing out that the chromaffin reaction of the leech neurons resembled more the reaction of enterochromaffin cells, which do not contain epinephrine (actually they contain 5-HT), than the reaction chromaffin cells of the adrenal medulla. Chromaffin cells have been also described in the earthworm CNS (301, 548).

No catecholamines have been found in the leech nerve cord (493). However, Ostlund (670) claims that the nerve cord of earthworm contains 1.4 µg epinephrine/g of wet tissue and 0.32 µg NE/g of wet tissue. More recently Myhrberg and Rosengren (cf. 627) found that NE amounts are 1 µg/g wet tissue in the earthworm nerve cord and 2 µg/g wet tissue in the brain, whereas dopamine seems to be completely absent. On the other hand, 5-HT is present in the CNS of leeches and earthworms; leech ganglia contain 6 µg/g of wet tissue and earthworm ganglia 10 µg/g of wet tissue (919).

Fluorescence histochemistry demonstrates that the above-mentioned six chromaffin cells of each leech segmental ganglion show a yellow fluorescence when studied with the Hillarp-Falck (see 245) technique (68, 225, 494, 544). There is general agreement among authors that these six neurons may contain 5-HT. Only in the periesophageal ganglia of the leech have green fluorescent neurons been observed (225). Recently a seventh fluorescent neuron has been described in *Hirudo* (580).

The "colossal" cells of Retzius, which form part of the group of yellow fluorescent neurons (225, 494, 544, 735), are two voluminous cells, easily distinguishable near the center of each ganglion. They are coupled by an electrotonic synapse (216, 348). The elegant work by Rude and her collaborators (735) has produced definitive evidence that Retzius neurons do contain 5-HT and that the amine is probably localized in submicroscopic cytoplasmic granulated vesicles. Microspectrofluorometric measurements from different regions of the colossal neurons demonstrate that their formaldehyde-induced fluorescence shows very similar excitation and emission maxima to those shown by model systems composed by formaldehyde-treated 5-HT in a dried albumin film (735). Moreover, extracts of 600 isolated colossal neurons subjected to thin chromatography resulted in a chromatogram with definite spots, one located at the same migration level and showing the same microspectrofluorometric properties as standard solutions of 5-HT (735). Furthermore, single Retzius cells were dissected out, extracted, and analyzed fluorometrically for 5-HT. An average quantity of  $3.8 \times 10^{-10}$  g of 5-HT per cell body was recovered (735). Submicroscopic granules of about 1000 Å average diameter may be visualized in the cytoplasm of the Retzius cells. They are centered by an irregular dense core and may well contain 5-HT, since they give a positive reaction when studied with the Wood (946) chromaffin reaction (735). Isolated Retzius

neurons may synthesize 5-HT from 5-HTP. Present methods cannot detect any synthesis of 5-HT from tryptophan by isolated Retzius neurons (380).

The Retzius neurons may be inhibited when 5-HT is applied either by dilution in the environment or by iontophoretic jets (502), but it is not known if this effect has some physiological relevance nor whether Retzius neurons actually release 5-HT through their endings and precisely where these endings are located.

Fluorescent neurons and varicose fibers, both bright yellow and green, have also been observed with the Hillarp-Falck technique (245) in earthworms (67, 69, 627, 734) and in polychetes (138). The yellow fluorescent neurons seem to predominate in the nerve cord (138, 627, 734), where fluorescent neurons may amount to  $\frac{1}{10}$  of the whole neuron population (734). Green fluorescent neurons are more abundant in the cerebral ganglia (138, 627).

A preferential localization of catecholamines in sensory cells and of 5-HT in motoneurons has been repeatedly postulated (138, 225, 627). Unhappily these speculations do not have any physiological support yet.

6) *GABA*. Evidence for a possible role of GABA as inhibitory transmitter at neuromuscular junctions of annelids is summarized in section II B. However, no direct evidence exists yet for the presence or synthesis of GABA in leech motoneurons or for GABA participation in synaptic mechanisms in the annelid CNS.

#### B. Neuromuscular Junctions

1) *Structural background*. Studies of neuromuscular transmission in annelids have been performed on only a few members of that phylum: leeches, land earthworms, and some sabellid marine earthworms. The longitudinal muscles of the earthworm are the best known annelid muscles from both an ultrastructural and electrophysiological point of view. These are obliquely striated muscles, the fibers being attached by desmosomes but not showing nexuses or tight junctions (421, 648). The muscle fibers show multiple innervation (see below). Different types of nerve endings have been observed: some contain clear synaptic microvesicles averaging 400 Å in diameter; others contain big granulated vesicles varying in diameter from 900 to 1800 Å (648). There is no specialization of the membrane or membrane densities at the level of the junction proper, where clumps of vesicles attached to the inner side of the presynaptic membrane suggest the localization of synaptic contact (648).

Electrophysiological data on the somatic muscles of the leech are not yet available, but pharmacological studies are worth describing here. Earthworm nerve-somatic muscle transmission has been elegantly analyzed by Kuriyama and his coworkers (371-374, 427-429) using microphysiological methods. It is likely that the properties of leech muscle resemble those of the earthworm.

2) *Excitation and acetylcholine*. Gaskell (300) and Pantin (677) expressed the view that excitation of leech muscle was similar to that of vertebrate skeletal muscle, and Fuhnrc (292) showed that ACh (in concentrations down to  $10^{-6}$  g/ml) produces a contraction of the longitudinal muscle of the leech body wall that is potentiated by eserine. These observations constituted the groundwork for development

of the well-known method of bioassay of ACh using strips of leech dorsal body-wall muscles (132, 614; see 594). These effects of ACh and anticholinesterase drugs were confirmed by many others investigating leech or earthworm muscles (9, 43, 87, 255, 753, 949, 954).

Gaskell (300) reported in 1914 that curare completely paralyzed the "voluntary" muscle system of the leech, and many investigators have since confirmed that dTC, as well as nicotine in high doses, blocked the effects of ACh (43, 256, 950, 954). Recent detailed pharmacological studies by Flacke and Yeoh (255, 256, 954) confirmed that ACh receptors of leech muscle are nicotinic.

Bacq and Copée (43) showed that both eserine and curare were less effective in blocking the muscle contraction evoked by electrical stimulation of the ventral nerve cord, but as a result of this stimulation a substance was released that was capable of inducing leech muscle contraction when applied by perfusion. This substance could only be recovered when the physiological saline used for its recovery was eserized. Bacq and Copée (43) showed that it was also capable of producing hypotension when injected in the cat, and they postulated that it was probably ACh.

Electrophysiological methods were first applied to study neuromuscular transmission in *Nereis* and *Harmothoe* (393). When impaled with microelectrodes, the membrane potential of the somatic muscles of both leech and earthworm is rather low, about -35 mv (371, 379, 907). This low  $E_m$  is due to the high permeability of the membrane to both  $\text{Na}^+$  and  $\text{K}^+$ . In a sodium-free environment the muscle fibers become hyperpolarized by 20–25 mv, and their conductance decreases 77% (371, 372). This high permeability of the membrane to  $\text{Na}^+$  is controlled by the  $\text{Ca}^{++}$  concentration in the medium. Diminution of the  $\text{Ca}^{++}$  content of the medium to  $\frac{1}{6}$  normal causes a 16-mv depolarization of the cell (372).

Leech and earthworm muscles discharge spikes that show a short overshoot and a rather large undershoot (372, 907). The spikes in earthworm longitudinal muscles may be evoked by mechanical, nervous, or direct intracellular stimulation. Spikes by direct intracellular stimulation may be observed in a  $\text{Na}^+$ -free medium or in the presence of TTX (372, 429), which blocks the nerve action potentials (372). Muscle action potentials recorded in  $\text{Na}^+$ -free Ringer depend on the external  $\text{Ca}^{++}$  concentration (429).

A) EXCITATORY JUNCTIONAL POTENTIALS. Stimulation of peripheral nerves in the earthworm evokes both EJP's and IJP's (373, 427). In the absence of nerve stimulation, intracellular recording may reveal a subthreshold noise composed of miniature EJP's and IJP's (427).

The mEJP's are better observed when  $\text{Na}^+$  is fully removed from the Ringer solution (427), which brings the resting membrane potential from -35 to -55 mv and increases the membrane resistance. In concentrations of  $10^{-6}$  g/ml dTC completely blocks mEJP's (427). Picrotoxin, which blocks both mIJP's and IJP's, does not affect either mEJP's or EJP's (427, 428). The amplitude and frequency of mEJP's vary from fiber to fiber. The frequency-distribution curve of the mEJP amplitudes agrees well with a theoretical Poisson distribution (427) and is skewed, indicating a diffuse innervation of the muscle fibers (427).

The reversal potential of the EJP's recorded from earthworm somatic muscle soaked in normal Ringer, calculated by extrapolation from the straight line relating the amplitude of the EJP's to the membrane potential, was estimated to be about zero (42). The calculated values of the reversal potential of the mEJP's were found to range between -10 mv and 0 mv (373, 427). Some of these measurements were taken in the presence of picrotoxin to avoid confusion with reversed IJP's. Changes in the K<sup>+</sup> concentration may also alter the  $E_{EJP}$ : lowering K<sup>+</sup> in the Ringer solution causes a shift of the reversal potential in the hyperpolarizing direction, whereas increasing K<sup>+</sup> causes an opposite effect. The change in the reversal potential of the EJP's produced by a 10-fold change in K<sup>+</sup> concentration amounts to 24.5 mv. External Ca<sup>++</sup> concentration also influences the value of the reversal potential of the EJP's: for a 10-fold change in Ca, it shifts 17 mv (427).

The excitatory transmitter produces a complex alteration of the membrane permeability, increasing simultaneously the permeability to Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>++</sup>. Modifying the external concentration of Cl<sup>-</sup> ions does not influence either the amplitude of the EJP's or their reversal potential (427). In experiments measuring both the  $E_{EJP}$  and  $E_{mEJP}$  in different ionic media, the ratio between the permeability coefficients of Ca<sup>+</sup> and K<sup>+</sup> was calculated from the constant-field equation; the Ca<sup>++</sup> permeability coefficient was 8 times higher than the K<sup>+</sup> permeability coefficient (427).

Earthworm ACh-perfused longitudinal muscle fibers have a decreased input resistance when bathed either in normal or in Na<sup>+</sup>-free Ringer; dTC blocks the ACh effect on membrane conductance and also blocks both the EJP's and the mEJP's (427). Prostigmine produces both an increase in amplitude and a prolongation of the EJP's (427).

All these findings support the conclusion of classical pharmacological work on leech and earthworm muscles postulating that ACh and the natural transmitter of excitation may well be one and the same.

b) SABELLID MARINE WORMS. Pharmacological studies by Nicol (646) in *Brachioma* showed a different behavior of ACh receptors in somatic longitudinal muscle of this marine worm. It was observed that contractions induced by ACh were not affected by dTC, but are depressed instead by atropine, which is unable to block leech and earthworm cholinergic receptors (42, 43). This observation was confirmed by Alvarez et al. (6) in *Sabellastarte magnifica*. Moreover, these authors report that ACh may have complex effects causing muscle contractions preceded or followed by muscle relaxations. To explain these results they postulate the existence of a complex population of muscle fibers in the body wall of *Sabellastarte*. Microphysiological work is necessary for a precise classification of the ACh receptors and a better understanding of the properties of the muscles of this worm.

3) *Inhibition and GABA*. The existence of an inhibitory innervation in annelid muscles is an important finding of recent microphysiological work (373, 427, 428).

Both IJP's and mIJP's may be recorded intracellularly from earthworm muscles (427); the former may be obtained by field nerve stimulation. Miniature IJP's appear at random, and their amplitude may vary from hundreds microvolts to 11 mv. They may be better observed in normal medium than the mEJP's (427).

The amplitude-frequency curve of the mEJP's is skewed and fits well a Poisson distribution, the skewing probably being an expression of multiple innervation of the muscle fibers (427). When the discharge of an IJP is correctly timed it may suppress the spontaneous spike discharge. In a normal medium the IJP's may reach amplitudes of 14 mv in some fibers (373).

The reversal potential for the IJP lies around -55 mv. The mIJP's may reverse between -55 and -60 mv. Reduction of the external Cl<sup>-</sup> concentration to 5.6 mm by replacement with D-glutamate (inactive on the membrane conductance) leads to a reversal of both the IJP's and mEJP's (373). The same effects were obtained when Cl<sup>-</sup> was replaced by SO<sub>4</sub><sup>2-</sup> or aspartate (373). Replacement of Cl<sup>-</sup> by D-glutamate changed only transiently the resting potential of the fiber (373). On the other hand, the value of the reversal potential of the mIJP's shifted only 25 mv for a 10-fold change in the Cl<sup>-</sup> concentration (427) instead of the 58-mv change expected from the Nernst equation. Since the K<sup>+</sup> content of the medium does not significantly influence the reversal potential of the IJP's, this behavior may only be explained by a change in the internal Cl<sup>-</sup> concentration. No direct evidence of this was obtained. If the reversal potential of the IJP were similar to the Cl<sup>-</sup> equilibrium potential (373),  $E_{Cl}$  would be 25 mv higher than the value of the resting potential. As in other invertebrate excitable cells (see sect. viA), it would be necessary to suppose the participation of some pumping mechanism to explain this distribution of Cl<sup>-</sup> ions (427).

GABA mimicks well the inhibitory transmitter action either when applied to the muscles cells by perfusion or by iontophoresis (327, 428). GABA increases the membrane conductance by increasing selectively the Cl<sup>-</sup> permeability, thus causing hyperpolarization of the muscle fibers. The reversal potential of this hyperpolarization is around -60 mv (327, 428), which is slightly different from the  $E_{IJP}$  but identical to  $E_{mIJP}$ . Moreover, changes in K<sub>o</sub> do not alter the reversal potential of the GABA response (327, 428).

The effect of picrotoxin completes the similarity of this inhibitory junction with the arthropod inhibitory junctions where GABA is very likely a transmitter (see sect. ivB and viB). Picrotoxin by itself may produce a decrease in the resting membrane conductance, probably by blocking Cl<sup>-</sup> permeability (428). It is difficult to know whether this is a direct effect on the Cl<sup>-</sup> channels or if it is actually due to a blocking of the effects of a spontaneous release of inhibitory transmitter, which would maintain a certain increased Cl<sup>-</sup> permeability level. Furthermore, picrotoxin blocks the increase in membrane conductance produced by GABA and also blocks completely the mIJP's and the IJP's (327, 428). At variance with what was found for the crayfish inhibitory junction by Takeuchi and Takeuchi (809), Ito et al. (428) claim that the action of picrotoxin would result from a competitive antagonism with GABA for the same receptor sites.

From all the above data it must be concluded that very likely GABA may be the chemical transmitter mediating inhibitory synaptic activities at the neuromuscular junctions of the earthworm. It would be interesting to complete this evidence with biochemical data and with experiments on both release and uptake of GABA.

4) *Monoamines.* Recent studies have shown the existence of catecholamines and serotonin in the CNS of leech and earthworm (see sect. IIIA). The presence of nerve endings showing granulated vesicles (648) may indicate some intervention of monoamines at the periphery. The pharmacological information is rather deceiving. Epinephrine may either excite or inhibit the muscles according to dose (669, 949; see 42). More recently Anderson and Fänge (9) found that epinephrine, norepinephrine, and tyramine elicited contraction of the body-wall muscles of earthworm and that the effects of epinephrine were blocked by phentolamine.

The situation for 5-HT is more or less the same. In the leech 5-HT relaxed the contractions produced by ACh or nicotine (753). In the earthworm some authors found a contracting effect (9), whereas others observed a blocking of sustained muscle contraction but no effect on the resting muscle or the phasic tension (374). In both cases the 5-HT concentration was  $10^{-5}$  g/ml.

In sabbellid worms 5-HT at concentrations of  $10^{-8}$  g/ml or higher causes the relaxation of body-wall muscle strips, although this effect not only does not antagonize the excitation caused by ACh but, on the contrary, potentiates the ACh contractions (6).

This repertorium of effects shows that the physiological role of monoamines on annelid muscles is open to research.

#### IV. CRUSTACEANS

##### A. Central Nervous System

The main information available on chemical transmitters in crustacean CNS is related to the chemistry of the excitatory and inhibitory motoneurons innervating skeletal muscle. In spite of an increasing knowledge of interneuronal functions and connections, firm evidence about the identity of the transmitters released at the CNS neuropile is lacking.

1) *Structural background.* Detailed descriptions of CNS morphology and structure are given in Bullock and Horridge's book (105). Functional organization and ganglion integration have been reviewed by Wiersma (930, 931) and Kandel and Kupfermann (452).

The bilateral symmetry of the segmental ganglia and the large size of many central neurons in both crayfish and lobster have facilitated the identification of nerve cells. Mapping of neurons was attempted early using classical histological techniques (711). In crayfish ganglia a combination of both morphological and electrophysiological methods has been used to locate the position of some interneurons and to trace the course of a high number of sensory fibers in the nerve connectives and in some peripheral nerves (477, 479, 933, 934).

An important step, as in similar studies in other invertebrate nervous systems, has undoubtedly been the introduction by Stretton and Kravitz (786) of a refined technique of tracing the geometry of the nerve cell by injecting the fluorescent dye Procion yellow intraneuronally. They showed the location and complexity of

branching of some lobster central neurons (786). Using the same technique it has been possible now to trace the precise localization of the somata and the distribution of axons and "dendrites" of the neurons forming each segment of the crayfish lateral giant axons (707) and of the crayfish motoneurons (176, 480).

A different type of neuronal mapping has been successfully achieved by Otsuka et al. (672). By combining electrophysiological and biochemical methods these authors could identify 21 neuronal somas corresponding to excitatory and inhibitory motoneurons in the abdominal ganglia of *Homarus* (see below).

The existing ultrastructural studies on crustacean nerve cord are mainly concerned with the giant axon system and especially with both the electrotonic septal contacts (357, 682) and the rectifying electrical synapses of the giant axons to the giant motor fibers (190, 357, 721, 722). Only a very recent study of the neuropile of the brain of the crab *Scylla serrata* gives some account of the structure of presumable chemical synapses in a crustacean CNS (749). Here small nerve terminals make contact with secondary, tertiary, and higher order branches of the motoneurons involved in the eye-withdrawal reflex. Pre- and postsynaptic densities and agglomeration of dense-core granulated vesicles 250–500 Å in diameter are the main features of these synapses, but sometimes mixtures of agranular and granular vesicles may be observed (749).

2) *Properties of neurons.* The electrophysiological properties of the giant axons of lobster and crayfish (see 440, 928) have been thoroughly explored (172, 173, 447, 835, 950). The giant axons consist of a series of segments formed in each ganglion by a contralateral neuron that has an inexcitable soma (707); these segments are in electrical continuity through electrotonic septal contacts (908). Resting membrane potentials of about –80 mv may be recorded with intracellular microelectrodes; this resting potential depends mainly on  $K^+$  (173, 835), but does not fit well the constant-field equation (173). Neither  $Na^+$ ,  $K^+$ , nor  $Cl^-$  is distributed in equilibrium across the cell membrane (903, 904). The giant-axon action potentials may be blocked by removal of  $Na^+$  from the environment. Voltage clamp of the fibers using the sucrose-gap method shows that the early inward current is carried by  $Na^+$  (447), but  $Ca^{++}$  also probably has some participation in the genesis of the spike (950).

Only recently some information about the membrane properties of neuronal somata in crustacean CNS has been obtained. It has been known that membrane resting potentials in crayfish and lobster neurons may vary between –50 and –80 mv (584, 798, 814, 964). In a neuronal population located at the rostral half of the 3rd abdominal ganglion of *Homarus americanus*, a rather uniform resting potential of about –53 mv is recorded (964). This value seems to arise from a Donnan distribution of  $K^+$  and  $Cl^-$  across the cell membrane, which is permeable even to rather large anions like  $SO_4^{2-}$  (964). Blocking of metabolic pumps by anoxia or ouabain does not alter the membrane potential (964).

It has been already mentioned that the somata of segmental giant-axon interneurons of crayfish are inexcitable (707). In many cases, like the motor giant neurons of crayfish abdominal ganglia or motoneurons of lobster, only an electrotonic potential can be recorded when different areas of the axon are activated

and changes in soma polarization do not affect the firing of the axons (672, 798). In other cases full impulses may be initiated by depolarizing the somata or impulses arising in axonal branches may be capable of invading the somata (479). Still another case observed is the existence of pacemaker neurons where endogenous spikes originating in the axon may invade the somata; these neurons may also show repetitive or oscillatory discharges (602, 700, 798).

The electrophysiological studies on the reflex activities in crayfish ganglia were initiated by Prosser (703) and reviewed by Wiersma (930, 931). It has become evident that synaptic activity may be recorded intracellularly, especially when the electrode tips are placed in elements of the neuropile, probably into fine "dendrites" or axonal branches (699, 909). However, EPSP's were observed when lobster motoneuron somata were impaled (672). Fast EPSP's and slow depolarizing PSP's may be recorded from giant motor fibers of the nerve cord of the crayfish when the cord is stimulated with a field electrode placed on the dorsal surface (294, 295). Sometimes these slow PSP's may appear "spontaneously" (295). The fast EPSP's are due to the activation of an electrical rectifying synapse linking the lateral giant axons to the giant motor fiber (294). Furshpan and Potter (295) found that the slow PSP actually corresponds to an IPSP since it reverses at a level only 7 mv more depolarized than the  $E_m$  and may depress the EPSP arising at giant motor synapses. A recent precise analysis of this IPSP has been done by Ochi (654). In giant motor fibers with resting potentials of about -63 mv, the slow IPSP's reverse around -53 mv.  $\text{Cl}^-$  appears to be the main ion generating the slow IPSP's, since removal of  $\text{K}^+$  does not affect  $E_{\text{IPSP}}$ . Replacement of 50% of the  $\text{Cl}^-$  content of the medium by propionate causes a shift of the  $E_{\text{IPSP}}$  of 7.5 mv instead of the 18-mv shift predicted by the Nernst equation (654). This behavior could be due to changes in  $[\text{Cl}]_o$ , while  $[\text{Cl}]_o$  is changed or to the fact that propionate ions are not properly impermeant.

3) *GABA*. The proof that GABA is involved in peripheral inhibition in crustaceans is most compelling (see sect. ivB, C) and has been confirmed by the experiments of Otsuka et al. (672) localizing the excitatory and inhibitory motoneurons in lobster ganglia and assaying for GABA content. The asymmetry of the excitatory and inhibitory axons with respect to their contents of GABA and the enzymes involved in its synthesis (glutamic acid decarboxylase) is fully described in section ivB. It also has been demonstrated at the level of the neuronal somata (380, 672, 698).

Otsuka et al. (672) identified by direct stimulation 18 excitatory motoneurons. Three inhibitory neurons acting on skeletal muscles were identified chemically by their GABA content and their character was then confirmed by electrophysiological methods (672). The excitatory motoneurons that innervate similar synergistic muscles appear clustered, whereas inhibitory neurons appear gathered together even if they direct their axons to different muscles (672).

Biochemical assay of isolated cell bodies of two of the inhibitory interneurons demonstrated that they contain around  $14 \times 10^{-3}$  M GABA and  $23 \times 10^{-3}$  M glutamate (672). These values may be altered in some way by the dissection techniques or by volumetric considerations (672). Excitatory motoneurons do not

contain enough GABA to be detected by the highly sensitive assay method used. Both excitatory and inhibitory motoneurons contain a similar quantity of glutamate (672). The concentration of GABA at the somata appears to be analogous to that observed in peripheral inhibitory axons (see 512, 698, and sect. IVB). More recent experiments by Hildebrandt et al. (380), using high-voltage paper electrophoresis to trace the incorporation of [ $^{14}\text{C}$ ]glutamate into neurons of lobster ganglia, demonstrate that GABA may be synthesized at the cell body of inhibitory motoneurons. The cell body of inhibitory motoneurons contains, as do the axons, glutamic acid decarboxylase (see also 353).

No information about the presence of GABA in other central neurons of crustaceans is yet available. The spontaneous activity of *Orconectes* ganglia does not seem much altered by GABA applications in high concentrations,  $\beta$ -alanine being much more effective in producing depression of activity (368, 369). However, GABA perfused in concentrations of  $3\text{--}5 \times 10^{-5}$  g/ml mimicks well the slow IPSP recorded at the giant motor fiber after field stimulation of the nerve cord (295, 654). GABA decreases the membrane resistance of the motor fiber by a factor of 5 (295). The effect of GABA shows an ionic basis similar to that of the IPSP. The reversal potential of GABA action shifts in the same way as the  $E_{\text{IPSP}}$  when  $\text{Cl}^-$  is replaced by propionate, and changes in  $[\text{K}]_o$  do not affect the conductance changes produced by GABA (695). Picrotoxin, which appears to increase spontaneous activity in crayfish ganglion (369), has been assayed neither on the slow IPSP nor on the GABA receptors. However, it seems possible that GABA may be involved in the genesis of these slow IPSP's. More work is necessary to explore further the possible role of GABA in crustacean central synapses.

4) *Acetylcholine*. Different elements of a cholinergic system have been detected in crustacean central nervous tissue (see 840). Variable quantities, between 10 and 90  $\mu\text{g/g}$  wet tissue of ACh, were early reported to be present in the nerve cords of *Homarus* (103, 503, 579, 628, 754, 917), *Cambarus* (213, 780), *Astacus* (14, 265), and *Carcinus* (906, 916). Choline acetylase activity has been demonstrated in homogenates of *Cancer borealis* ventral cord ganglia that may synthesize 200  $\mu\text{g/g}$  tissue per hour (213). ACh synthesis has also been demonstrated by using homogenates of *Carcinus* ganglia (905, 906). A cholinesterase inhibited by eserine was detected in the central ganglia of *Homarus* (103, 579, 915), *Astacus* (265), and *Carcinus* (906, 918). Large amounts of cholinesterase, probably acetylcholinesterase, may be demonstrated when histochemical methods are applied to lobster ganglia. The enzyme appears to be localized both between and inside the sheath cells and at the neuropile, but only small amounts are located intraneuronally (586).

Early studies of ACh action on crustacean ganglia have been mainly devoted to the effects of cholinergic drugs on the synapses of giant axons to giant motor fibers. No effects of these drugs were observed (755, 935). These synapses were later found to be electrically transmitting junctions (294). On the other hand, report on the effect of cholinergic drugs on spontaneous activity recorded from whole ganglia are rather contradictory. A typical example is eserine, which has been reported as either increasing or blocking the spontaneous activity (369, 424,

755). No microphysiological studies are yet available on direct effects of cholinergic drugs on neurons or synapses.

A piece of information recently confirmed may give some suggestion about cholinergic systems in crustaceans: the 1st and 2nd roots but not the 3rd root of the abdominal ganglia of the lobster may synthesize ACh when incubated in the presence of the required substrates (269, 380). These 1st and 2nd roots contain both efferents and afferent axons, whereas the 3rd root contains only afferent axons (418). This finding suggests that the sensory neurons are perhaps cholinergic. Preliminary evidence shows that the stretch receptor neurons are capable of synthesizing ACh (380).

Barker et al. (44a) recently confirmed a parallelism between the distribution of acetylcholine transferase and the proportion of sensory fibers in lobster peripheral nerves. Moreover many sensory organs, among them the stretch receptor organs, incorporate radioactive choline into ACh (44a). They identified synaptic contacts between sensory fibers and central neurons endowed with ACh receptors. The EPSP's recorded at such synapses are partially blocked by dTC or atropine (44a).

5) *Monoamines.* Lancaster (548) described the existence of "chromaffin" cells that probably contain an epinephrine-like substance in crayfish nerve cords (see 626).

Studies using the Hillarp-Falck technique (245) have been recently performed on the central nervous system of *Astacus* (234) and on the stomatogastric ganglion of *Homarus* (668).

In *Astacus* both green and yellow fluorescent neurons have been visualized. The green fluorescent neurons would contain NE and DA and the yellow ones would contain 5-HT. Reserpine causes the disappearance of both types of fluorescence (234). Green fluorescence was also found at the neuropile in varicose fibers and in large beaded axons (234). In the stomatogastric ganglion of *Homarus* all the fluorescent cells were yellow-green, and this fluorescence also disappeared under the effects of reserpine (668). In both cases nialamide, a MAO inhibitor, does not alter the fluorescence much. Pharmacological evidence on the possible role of monoamines is nonexistent.

#### B. Neuromuscular Junctions

Richet (713) was first to suggest the existence of peripheral inhibition in crustacean muscle. The now classical work of Biedermann (70), Mangold (574), Hofmann (390), and others demonstrated that crustacean muscles have a complex innervation involving both motor and inhibitory nerves (174, 506, 529, 678, 679, 889; see reviews in 293, 459, 929).

Identification of the neuromuscular transmitters in *Crustacea*, especially the inhibitory transmitter, has been rather successfully achieved. Compelling biochemical and physiological evidence has been gathered in favor of a transmitter role for GABA in neuromuscular inhibition. The evidence in favor of glutamate as

a transmitter of junctional excitation, though not so strong, is highly suggestive. It is worthwhile keeping in mind here that the results obtained in the study of insect neuromuscular junctions (sect. vB), are complementary in many aspects. The neuromuscular mechanisms involved in all the arthropods thus appear to be the same.

1) *Structural background.* Excitatory and inhibitory axons run parallel in the nerves (528, 529, 885, 889) and end in a widely distributed manner, forming a large number of junctions (28, 29, 208, 250, 885, 927). For instance, the motor neuron that supplies both the cheliped opener and stretcher muscles of the crayfish makes 50 endings on each of 1200 muscle fibers (73). Generally the crustacean muscle fibers receive 1–4 excitatory axons and 1 or 2 inhibitory axons or inhibitory innervation may be absent. The pattern of innervation may vary according to individual muscles and species [discussed in previous reviews (25, 929)]. The excitatory axons have been recognized as fast and slow axons according to the modalities of stimulation required to obtain a muscle contraction (see 929). The muscles also may show fast or slow mechanisms of contraction, and accordingly they present different structural and electrophysiological properties (145, 244; see 25).

Electron-microscopic studies of crayfish and crab muscles reveal that the axons may end penetrating in grooves, invaginated clefts, or deep invaginations of the muscle cell surface (29, 409, 436). The synaptic membranes forming the junction are separated by a 200-A cleft, the presynaptic regions containing mitochondria and the synaptic vesicles attached to "active" points. No difference has been observed between endings supposed to belong to fast and slow excitatory axons in muscles that do not receive an inhibitory input (145, 409).

After failure to recognize ultrastructural differences between excitatory and inhibitory axon endings (88, 145, 409) some recent papers claim to have achieved such a differentiation. In the leg muscles of crayfish, two types of endings could be distinguished according to the vesicle population they contain (28, 30). In one type of terminal the synaptic vesicles would seem to be larger and apparently 90% of the electron-lucent vesicles inside them appear with round profiles. In the second type of ending the vesicles seem to be smaller and only some 45% of them are round, with the remaining showing an ellipsoidal shape (28). A thorough analysis of these apparent differences shows that they are statistically significant, especially when the ratios between the maximal and minimal vesicle diameters in each population are compared (30). The function of each one of this type of ending was inferred from the structure of axo-axonic synapses found in muscles presenting presynaptic inhibition that are supposed to constitute the morphological substratum of this type of inhibition (see below). These axon-to-axon synapses (28, 30) are 10–20 times less frequent than the nerve-to-muscle junctions. They always appear formed by a terminal containing the smaller vesicles, making contact with a specialized postsynaptic membrane of another nerve terminal containing predominantly the larger and round vesicles. Since the presynaptic ending generates the presynaptic inhibitory phenomena it is easy to qualify as excitatory or inhibitory each of the endings participating in such an axo-axonic synapse (30).

An independent finding in support of such hypothesis comes from studies on the structure of the muscle fibers that form the stretch receptor organs in crayfish (510, 631). Using glutaraldehyde fixation, it was found that two types of endings make contact with these muscles: terminals containing flattened ellipsoidal vesicles and others containing round vesicles. The endings containing flattened vesicles are the only ones to synapse with the stretch receptor neuron. Since this neuron receives exclusively inhibitory inputs (see sect. IV C), it may be concluded that the endings containing flattened vesicles are inhibitory and the ones presenting round vesicles may be excitatory (510, 631, 864; see sect. IV C).

2) *Junctional transmission.* A discussion of the particular membrane properties of different regions of crustacean muscle membranes is out of the scope of this review (see discussions in 366 for crab muscles, 661 for crayfish muscle).

Stimulation of excitor axons produces depolarizing deflections of the muscle membrane potential, the EJP's that may be recorded intracellularly from any point of the muscle fibers and extracellularly from multiple foci (250, 529). In dually innervated motor fibers, stimulation of fast and slow axons produces EJP's of different amplitudes and time courses (250, 410). The influence of slow and fast axons on muscle contraction has been discussed in previous reviews (25, 293, 459, 929).

Nerve stimulation of crayfish leg muscle generally causes a local regenerative response that is not overshooting (248). Removal of  $\text{Na}^+$  of the environment and its replacement by quaternary ammonium salts causes the conversion of the local response to a full overshooting spike (248) generated by an increase of membrane permeability to  $\text{Ca}^{++}$  ions (247). In barnacle muscles, which also normally show a local regenerative response,  $\text{Ca}^{++}$ -dependent spikes may be discharged after the fibers are injected with chelating agents (345, 350). Tetrodotoxin and saxitoxin do not alter the Ca spike of crustacean muscle fibers (674). Muscle fibers giving full overshooting spikes in normal conditions have been observed in both crayfish and crab (see 25).

By timing differently the stimulation of an inhibitory axon with respect to the stimulation of an excitatory axon, Marmont and Wiersma (580) distinguished two classes of inhibition in crustacean muscle. In the first type, which they called "supplemented" inhibition, the excitatory electrical activity caused by the stimulation of the excitor nerve was attenuated, whereas in the second type of inhibition, which they called "simple" inhibition, they observed a sort of uncoupling of the process linking excitation and contraction without any apparent change in the electrical manifestations of excitation. Kuffler and Katz (528) confirmed these observations, denoting  $\alpha$ -inhibition the "supplemented" inhibition and  $\beta$ -inhibition the "simple" inhibition. These authors also demonstrated that the  $\beta$ -inhibitory action caused a reduction of the amplitude of the EJP's recorded extracellularly at crayfish neuromuscular junctions, the reduction depending on the relative time of arrival of the inhibitory and the motor impulse to the junctions.  $\alpha$ -Inhibition was thus interpreted as due to the release of an inhibitory transmitter that would act by "curarizing" the excitatory transmitter receptors of the muscle membrane (249, 528), whereas  $\beta$ -inhibition was interpreted as a possible direct action of the

inhibitory nerve on the contractile process since complete mechanical inhibitions could be obtained without apparent alteration of the EJP's (528, 578). Later work disclosed the existence of two different mechanisms of inhibition mediated by branches of the same inhibitory axon: a postsynaptic mechanism resulting from an action of the inhibitory transmitter on the membrane conductance of the muscle membrane (84, 249) and a presynaptic mechanism due to an action of the inhibitory transmitter on the excitatory synaptic endings (210). The postsynaptic mechanism may account for phenomena previously described either as  $\alpha$ - or  $\beta$ -inhibition, whereas many  $\beta$ -inhibitory mechanisms were probably presynaptic in character (see 26). The relative importance of each type of inhibitory mechanism may vary according to different muscles (32, 478).

Intracellular recording shows that the EJP's are reduced in amplitude under the influence of inhibitory impulses and their rate of decay becomes faster, even if the inhibitory impulse arrives during the EJP falling phase (249). However, the most interesting finding is that generally the stimulation of the inhibitory axon does not cause any "overt" effect on the membrane potential of the muscle fiber (84, 246, 249, 342, 411), although small deflections may be seen. When the membrane potential is increased or decreased artificially by injecting current through a second intracellular micropipette, an effect of the inhibitory impulse on the membrane potential becomes apparent as either a depolarization or a hyperpolarization according to the level at which the membrane potential has been driven (84, 249, 342). These observations were interpreted first as resulting from an increase in the membrane permeability to ions ( $\text{Cl}^-$  or  $\text{K}^+$ ) "whose movement normally served to maintain and restore the resting potential" (249). The duration of this permeability change responsible for the IJP's is shorter than the duration of the potential change and lasts only as long as the rising phase of the IJP's (84, 249, 342). The ionic mechanism of both EJP's and IJP's is considered later.

3) *Inhibition and GABA.* GABA was discovered at the beginning of the century (1) and was first detected in mammalian brain by Awapara et al. (39), Roberts and Frankel (720), and Udenfriend (865) (see review of early data in 233, 717).

Florey (258, 259) found that the brain and spinal cord of mammals contained an extractable substance that in low concentrations blocked crustacean neuromuscular transmission and inhibited the crayfish stretch receptor neuron (259). The same substance also showed other effects: such as inhibition of spontaneous contractions of isolated intestine and slowing of neurogenic heartbeat (260, 261). The inhibitory substance (factor I) was present only in nervous tissue and it was dialyzable and thermostable. The effects of factor I could be dissociated from the action of other presumptive transmitters also present in brain tissue (275). Some blocking effects of factor I may be observed on vertebrate mesenteric and stellate ganglia, but not on the superior cervical ganglion (274). Bazemore, Elliott, and Florey (53) submitted a large amount of factor I obtained from beef brain to fractional crystallization (see also 232), obtaining a crystalline material that could be identified as GABA and was shown to be the most active component of the extracts when assayed for inhibitory action on crustacean stretch receptor neurons.

In spite of this evidence, it was repeatedly claimed that GABA did not account

for all the pharmacological actions of factor I (392, 595, 596). Moreover, it was claimed that factor I and not GABA was present in extracts of crab and lobster peripheral nerves, where it could account easily for the inhibitory action of these extracts on stretch receptor neurons (269, 270). Moreover, Florey and Biederman (269), having calculated that the inhibitory action of an extract of inhibitory nerves would be equivalent to the effect of 30,000 µg GABA/g of nerve wet weight, claimed that therefore GABA could hardly be the inhibitory transmitter.

However, even if GABA could not account for many of the actions of factor I (see 597) the arguments against its transmitter role in peripheral inhibition in crustaceans mainly arose from the assay techniques used. These techniques undoubtedly were not sensitive enough to detect GABA correctly (see discussion in 266). Very careful and precise studies by Kravitz and his collaborators at Harvard have shown that GABA is present in crustacean inhibitory neurons, where it is synthesized and accumulated and then released at peripheral endings located on exoskeletal muscles fibers.

In these studies three main inhibitory substances were first extracted from lobster CNS: GABA, taurine, and betaine (205). In the peripheral nerves of crab and lobster ten inhibitory compounds were found; of these GABA, taurine, and betaine accounted for most of the inhibitory activity as assayed on crab muscle (515, 516, 519). The other compounds are  $\beta$ -alanine, alanine, homarine, glutamine, aspartic acid, and two other nonidentified substances (514). These substances were identified by electrophoresis and paper chromatography, and GABA was identified using a specific assay procedure (205). The GABA content was found to be proportional to the number of inhibitory axons in the material (516). Furthermore, GABA is concentrated in a proportion of 100:1 in peripheral inhibitory axons and neurons (516, 518): when the GABA content is assayed in lobster leg inhibitory axons isolated from the other excitatory and sensory axons running in the same nerve, it appears that GABA constitutes 0.5% of the wet weight of the inhibitory axons but it cannot be detected in isolated excitatory axons (516, 518). GABA accounts for 50% of the inhibitory activity of the inhibitory nerve extract, the remainder being due to the other eight inhibitory compounds already mentioned. The distribution among the different axons of  $\beta$ -alanine is not clear. These findings could be confirmed in three types of inhibitory axons (518), and in all cases the inhibitory axons contained 100 times more GABA than the excitatory axons, with concentrations amounting to 0.1 M (353, 517).

The same difference in concentration is found at the central ganglia between the cell bodies of identified inhibitory and excitatory neurons innervating skeletal muscles (672). The inhibitory neuronal somatas contain 100 times more GABA than the somatas isolated from identified excitatory motoneurons. Glutamate is present inside both types of neurons (see sect. IV A).

A) METABOLISM OF GABA IN CRUSTACEAN NERVOUS SYSTEM. The metabolic pathway for the synthesis and metabolism of GABA in the crustacean nervous system was found by Kravitz and his colleagues (see reviews in 512, 513) to be very similar to that in the mammalian nervous system (see 47, 719). The pathway was determined with enzyme extracts prepared from the CNS of the lobster

*Homarus americanus*. The synthesis and degradation of GABA are carried out by three enzymes: 1) L-glutamic acid decarboxylase, which synthesizes GABA from glutamate by removing the  $\alpha$ -carboxyl group and releasing CO<sub>2</sub> (511, 618); 2) GABA-glutamic transaminase, which converts GABA to succinic semialdehyde (SSA) in the presence of  $\alpha$ -ketoglutarate (354); and 3) a succinic aldehyde dehydrogenase, which converts SSA to succinic acid (355).

The glutamic decarboxylase extracted from lobster has a pH optimum of 8.0. Potassium and  $\beta$ -mercaptoethanol are essential for its activity. Glutamic decarboxylase is inhibited by its product GABA through a mechanism resembling competitive inhibition (618). Excitatory and inhibitory axons also show great differences in glutamic acid decarboxylase activity. It has been found to be 100 times higher in the inhibitory than in the excitatory fibers, which show activities near the limits of sensitivity of the assay method (353, 517). In the presence of 0.1 M GABA the enzyme activity in the axon homogenate is reduced by 65%, whereas the same conditions may cause a 90% inhibition of the activity of an enzyme solution (618).

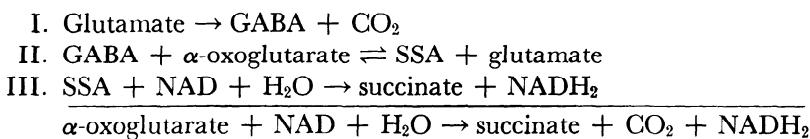
An important aspect of this enzyme inhibition by GABA is that it may possibly serve as a regulatory mechanism in the selective accumulation of GABA in inhibitory neurons, i.e. regulating the final level of GABA. It may be supposed that when GABA concentration falls in the inhibitory neurons, the inhibition of the enzymic activity also disappears and GABA may again accumulate (618). No subunit structure of the decarboxylase has been described yet, and the sedimentation coefficient of the enzyme could not be changed by any of the agents that affect the enzyme activity. In crude homogenates of the lobster CNS 90% of the enzyme is found in the soluble subcellular fraction. No indication exists that GABA or its synthesizing enzyme is related to a particulate component of the nerve endings.

GABA-glutamic transaminase isolated from lobster CNS does not appear to be different from other transaminases (354). The enzyme is stimulated by pyridoxal phosphate and inhibited by carboxyl reagents (hydroxylamine, semicarbazide). The most potent inhibitor of the enzyme is amino oxyacetic acid. The kinetic properties of the enzymes for the forward reaction are consistent with a mechanism in which each substrate, GABA and  $\alpha$ -ketoglutarate, react alternately with the enzyme (354). The enzyme may be inhibited by high concentrations of GABA and  $\alpha$ -oxoglutarate. Both *p*-hydroxymercuribenzoate and *N*-ethylmaleimide also inhibit the enzyme. Other amino acids may serve as substrates for the enzyme, such as  $\beta$ -alanine and  $\gamma$ -aminovaleric acid, but not as well as GABA (354). It is very unlikely that GABA transaminase could be involved in GABA inactivation in the case of GABA release by the nervous impulse at synapses in the same way as acetylcholinesterase acts on ACh. The requirement of the enzyme for  $\alpha$ -oxoglutarate makes its localization at the outer surface of the pre- and postsynaptic membranes rather unlikely. Potter (cf. 354) did not find any prolonging effect of transaminase inhibitors such as amino oxyacetic acid or hydroxylamine on neuromuscular transmission in the lobster. Uptake by the nerve ending appears to be a better mechanism of inactivation of GABA (see below). GABA-glutamic

transaminase is found in both excitatory and inhibitory axons, but about 50% more enzyme activity is detected in the inhibitory axons (353).

After GABA becomes metabolized to succinic semialdehyde (SSA) by the transaminase, a second step converts SSA to succinate. The lobster enzyme is similar to mammalian brain enzyme (355), having an alkaline pH optimum of about 9.0 and requiring the presence of a thiol, being almost inactive in its absence.  $\beta$ -Mercaptoethanol and dithiothreitol (DTT) activate the enzyme equally but the highest activity is obtained when NAD is present during preincubation. Succinic semialdehyde has no effect by itself on the enzyme. Unlike the mammalian enzyme, the lobster is inhibited by NaCl. It can also be inhibited by arsenite.

The GABA metabolic pathway in the lobster nervous system may be summarized as follows (513):



b) **GABA RECEPTORS.** After the discovery by Bazemore et al. (53) that inhibitory effects of factor I on crayfish stretch receptor neurons were due to GABA, this amino acid was assayed on crustacean muscle and found to mimic the inhibitory transmitter in suppressing the muscle contraction elicited by the stimulation of the excitor nerves (92, 715, 884). Boistel and Fatt (84) and Grundfest et al. (342) observed that GABA changed the membrane permeability of both crayfish and lobster muscles in the same way as the inhibitory transmitter (see below).

It was due to the elegant work of Takeuchi and Takeuchi on the crayfish leg muscle that very precise information emerged on the properties of GABA receptors. When GABA is applied iontophoretically from a micropipette on the surface of the fibers of the abductor muscle of the dactylopodite of *Cambarus* walking leg, only circumscribed spots of the membrane show sensitivity to GABA jets (804, 805; see 808). The resulting GABA responses are generally depolarizing potentials slower than the glutamate potentials elicited from the same spots (see below), and when the application is sustained the falling phase of the responses is prolonged. Thus long-lasting iontophoretic injections in this preparation do not permit observation of receptor desensitization (804). GABA receptors are located in the same spots as glutamate receptors and these regions also coincide well with the inhibitory neuromuscular junctional areas as revealed by monitoring both the IJP's and the GABA responses by simultaneous extracellular and intracellular recording (804, 808). Therefore, the GABA- and glutamate-sensitive regions, as well as the inhibitory and excitatory junctional regions, are side by side (808). GABA and glutamate receptors are structurally different since no crossed desensitization can be elicited between them (804). Furthermore this lack of competition between GABA and glutamate for the same receptor is confirmed by voltage-clamp experiments recording the current generated by glutamate iontophoretic application. The iontophoretic application of GABA does not alter the current generated by the L-glutamate response (805).

GABA effects on the membrane conductance, on the EJP's, and on muscle contraction of crayfish muscle were shown to be effectively blocked by picrotoxin at concentrations higher than  $10^{-5}$  M (340-342, 883, 884). Picrotoxin itself does not cause changes in membrane conductance and does not cause muscle contraction (341, 343, 884). Picrotoxin also blocks the IJP's, without affecting the inhibitory nerve conduction (341, 342, 809, 883, 884). Structural analogs of GABA such as  $\beta$ -alanine,  $\beta$ -aminobutyric acid, glycine,  $\delta$ -aminovaline,  $\epsilon$ -aminocaproic acid, and guanidinoacetic acid all behave as GABA agonists on the muscle postsynaptic membrane, increasing the conductance and reducing the EJP's (199). Other drugs, like  $\beta$ -guanidinopropionic acid ( $\beta$ -GP) and  $\beta$ -guanidinobutyric acid, do not alter the postsynaptic membrane permeability, but may have some pre-synaptic effects. At the postsynaptic membrane  $\beta$ -GP may block the effects of GABA on the conductance and also block the IJP (199).

Takeuchi and Takeuchi (807) established dose-response curves relating GABA concentrations perfused to an in vitro preparation of abductor muscle fibers of the dactylopodite of *Cambarus* leg with the corresponding changes induced in the membrane conductance per unit length of the fiber. They concluded that GABA may combine with its receptors following a process described by the Langmuir absorption isotherm and that the increase in membrane conductance is linearly proportional to the number of receptors occupied by GABA. From equations derived from Michaelis-Menten kinetics, Takeuchi and Takeuchi (807, 809) calculated that two molecules of GABA must combine with a single receptor in order to produce a membrane conductance change. This hypothesis has been challenged by Feltz (252), who finds that the dose-response curves may be different when they are drawn from experiments in which, instead of perfusing with progressively increasing concentrations of GABA, the perfusion of each individual GABA concentration is separated by a thorough washing of the preparation. This procedure produces marked changes in the slope of the dose-response curves relating GABA concentrations to conductance change, and from Hill's plots it may be calculated that the conductance change would result from the combination of four molecules of GABA with a single receptor (252). The change in slope due to the modification of the experimental procedure is attributed to a desensitization of GABA receptors appearing when the increasing concentrations are not sufficiently separated by washing periods (252).

These quantitative methods also have been applied to the analysis of the action of GABA antagonists on the GABA receptor. Picrotoxin appears to act as a noncompetitive antagonist (809) and to be more effective in low-Cl<sup>-</sup> media (809). This noncompetitive action could be due to an interference of picrotoxin with the ionic permeability mechanisms (the "ionophore") (809). On the contrary,  $\beta$ -GP was found to be a competitive antagonist of GABA for the same membrane receptors (252).

c) DESENSITIZATION OF GABA RECEPTORS. It has been mentioned previously that desensitization of GABA receptors in crayfish muscle is not apparent when GABA is applied iontophoretically by using long-lasting currents but becomes evident when the muscle is submitted to successive perfusions of increasing concentrations (252).

Desensitization phenomena may also explain the rather contradictory evidence reported in the last 10 years on GABA action on crab muscles (236). On muscle fibers of the opener or closer muscles of *Cancer borealis*, GABA was reported to cause in a few seconds an 80% reduction of the amplitude of the EJP and a marked decrease of membrane resistance (226). However, in muscle fibers of *Cancer borealis* or *C. magister* it was claimed by different authors that, in spite of reducing the EJP, GABA affected the membrane conductance only slightly or not at all (2, 273, 411). A proposed explanation of these contradictory results is heterogeneity of the crab muscle fibers, which may or may not receive inhibitory input and thus have GABA sensitivity (24, 26). Actually the explanation seems to be that GABA changes the effective resistance of *C. borealis* muscle fibers, but this conductance alteration is very transient because of the rapid development of intense desensitization phenomena (236).

D) IONIC BASIS OF IJP AND GABA RESPONSES. Boistel and Fatt (84) first showed that the inhibitory transmitter changes the crustacean muscle membrane permeability specifically to  $\text{Cl}^-$  ions. In many crustacean muscles the reversal potential level for the IJP's ( $E_{\text{IJP}}$ ) almost coincides with the resting potential (84, 342, 384, 805). In the opener claw muscle of *Astacus fluviatilis* (84) removal of  $\text{K}^+$  from the saline increases the resting potential but causes a very slight change of  $E_{\text{IJP}}$ . However, replacement of  $\text{Cl}^-$  by pyroglutamate or acetylglycine, which decreases the resting potential by 10–20 mv, shifts the  $E_{\text{IJP}}$  in such a way that depolarizing of the muscle fiber does not reverse the IJP even when the membrane potential is artificially driven near the zero potential level (84). Increase of the external  $\text{K}^+$  concentration up to 20 mm in the presence of  $\text{Cl}^-$  decreases the resting potential (30 mv in 20 mM  $\text{K}^+$ ), but again the  $E_{\text{IJP}}$  is almost not affected by this  $\text{K}^+$  alteration. These data clearly indicate that the inhibitory transmitter selectively increases the membrane permeability to  $\text{Cl}^-$  ions. This selectivity for the anion is attributed by Boistel and Fatt (84) to the possible presence of charged pores in the membrane.

In lobster muscles the inhibitory junctional potentials also arise from an increase in  $\text{Cl}^-$  conductance (342, 625). In the stretcher muscles of the walking leg of *Homarus americanus*  $E_{\text{IJP}}$  was recently found to depend on the electrochemical gradient for a "mobile" fraction of the intracellular  $\text{Cl}^-$  (625). The  $E_{\text{IJP}}$  is the same when measured singly or on fused repetitive IJP's. The mechanisms regulating  $E_m$  and  $E_{\text{IJP}}$  are different. There is no contribution of  $\text{K}^+$  permeability to the genesis of the IJP except through a slow redistribution of  $\text{Cl}^-$  when  $\text{K}^+$  concentration in the medium is changed (625). As in the crayfish, the  $E_{\text{IJP}}$  in lobster muscles is markedly changed by alterations in the  $\text{Cl}^-$  gradient, whereas it appears rather unaffected by an alteration of  $\text{K}^+$  concentration in the external medium. When the external  $\text{Cl}^-$  concentration is varied between 50 and 500 mM, the curve resulting by plotting  $\log (\text{Cl})_o$  against the  $E_{\text{IJP}}$  deviates from the Nernst theoretical straight line for concentrations below 300 mM. Since changes in the cations do not affect the  $E_{\text{IJP}}$  it is difficult to explain this deviation from the Nernst equation (625). As already mentioned, there is also some contradiction between the values of the internal  $\text{Cl}^-$  concentration calculated from the Nernst equation and the data obtained by chemical analysis (212). By the first method a value of

24 mM may be obtained (625), whereas average analytical values are around 90 mM/kg cell water. Only a fraction of the internal Cl<sup>-</sup> would then be free, and since Cl<sup>-</sup> appears not to be at equilibrium at  $E_m$ , the existence in lobster muscles of some Cl<sup>-</sup> pumping mechanism to maintain the concentration out of equilibrium has been postulated (625).

GABA and the inhibitory transmitter produce the same selective change in Cl<sup>-</sup> permeability of the postsynaptic membrane in crayfish and lobster muscles (84, 342, 625, 804, 805, 807, 810). In the abductor muscles of the walking leg dactylopodite of *Cambarus*, the changes produced by  $2 \times 10^{-4}$  M GABA solutions on the membrane conductance per unit length were studied in the presence of Cl<sup>-</sup> or when Cl<sup>-</sup> was partially or totally replaced in the saline by a series of organic and inorganic anions (807). The conductance change caused by GABA decreases linearly as Cl<sup>-</sup> is replaced by impermeant ions like methylsulfate or propionate (807). The inhibitory postsynaptic membrane appears impermeable to cations and shows a variable degree of permeability to different foreign anions that follows the order (807, 810):



Changes in pH, using tris maleate buffers, affect the Cl<sup>-</sup> conductance change produced by GABA: increase in pH causes a diminution of the Cl<sup>-</sup> permeability alteration by GABA, whereas the decrease of pH enhances the GABA-induced conductance change (810). Variation in divalent cations does not modify the effects of GABA on the Cl<sup>-</sup> conductance (810).

Somewhat different results have been obtained in lobster muscle fibers by Motokizawa et al. (625). Instead of studying the effect of GABA on membrane conductance, as in the crayfish, these authors analyzed the changes introduced in the  $E_{\text{IP}}$  by the replacement of Cl<sup>-</sup> by different anions. The inhibitory junctional membrane of the stretcher muscles of the walking legs of *Homarus* appears more permeable to some foreign anions in the order: NO<sub>3</sub><sup>-</sup> > SCN > Br > Cl<sup>-</sup>. The inhibitory membrane is impermeable to BrO<sub>3</sub>, isethionate, and methylsulfate and slightly permeable to acetate and propionate.

There are also some differences between the anionic permeability of crayfish inhibitory postsynaptic muscle membranes and that of the motoneurons (see 214). However, the motoneuron membrane is also impermeable to BrO<sub>3</sub>. Snail neuronal postsynaptic membranes resemble crayfish membrane in their permeability to BrO<sub>3</sub> (498).

When  $\frac{1}{4}$  or  $\frac{1}{2}$  the Cl<sup>-</sup> in the saline bathing a crayfish muscle is substituted for one of the foreign anions, an interaction between different anions present in the medium modifies the membrane conductance (810). This confirms the data on anion selectivity and pH effects on the inhibitory membrane and gives strong support to the idea of Boistel and Fatt (84) that the inhibitory membrane is charged positively. Anions would penetrate by interacting with the charged sites (810).

Another interesting recent finding of Takeuchi and Takeuchi (811) is that the inhibitory postsynaptic membrane of crayfish muscle varies its anionic selec-

tivity according to the GABA concentration in the medium. For instance, in low concentrations of GABA the conductance change in a solution where  $\text{Cl}^-$  is partially replaced with  $\text{NO}_3^-$  or  $\text{I}^-$  is larger than in control solution containing  $\text{Cl}^-$ , whereas the membrane conductance change produced by a higher GABA concentration is more important in solutions where  $\text{Br}^-$  and  $\text{CNS}^-$  have replaced  $\text{Cl}^-$  than in the  $\text{Cl}^-$  control solutions.

E) PRESYNAPTIC INHIBITION AND GABA. Apart from the already described effects on the postsynaptic membrane, the inhibitory transmitter also acts on the excitatory presynaptic endings (207, 210, 341, 805, 806).

Dudel and Kuffler (207, 208, 210) have carefully analyzed the mechanism of presynaptic inhibition. It has long been known that when the inhibitory inputs are timed to arrive at the junction 1–6 msec before the arrival of an excitatory input, the EJP thus resulting will appear decreased in amplitude (528, 578). These effects were analyzed by Dudel and Kuffler (208, 210) paying special attention to the mechanisms of transmitter release.

Intracellular recording from fibers of the abductor muscles of the walking leg dactylopodite permits recording of practically all the EJP's produced by the diffuse excitatory endings (208, 250). In the same way mEJP's may be intracellularly recorded in any point of the crustacean muscle fiber (73, 208, 211, 710, 805). They are recorded as small deflections that show the same time course as the EJP, appearing at random at frequencies of 1/sec, which may be altered by excitor axon stimulation or an increase of the osmolarity of the environment (208, 211). High  $\text{Ca}^{++}$  concentrations, which apparently block the excitatory axon conduction, do not affect the mEJP frequency. The EJP's recorded from individual junctional areas with a focal extracellular microelectrode are quantal in nature (208). They are composed of discrete units that may be released according to a certain probability when an action potential arrives at the excitor axon endings, since the amplitude histograms of the EJP's recorded extracellularly from individual junctions may be well described by the Poisson theorem (208; for recent results that do not fit the Poisson theorem see 31, 73). The histograms show different peaks, whose amplitudes are multiples of a quantal unit with an amplitude similar to mEJP's recorded extracellularly (208). In an individual junction these extracellularly recorded mEJP's show low frequencies of 1/min (208). The quantal numbers of individual EJP's in crayfish are remarkably low, at variance with the values observed in transmitter release in vertebrates, but the difference is nearly made up by the multiplicity of innervation in *Crustacea* (73).

When well-timed inhibitory and excitatory inputs arrive at junctions to cause a decrease of the EJP's (see above), it is possible to observe that the reduction in the EJP's in these cases is not due to a postsynaptic effect but to a presynaptic influence of the inhibitory axon on the excitatory process decreasing the number of quanta released by the excitatory ending without alteration of the quantal size (210). Therefore, these effects are due to a decrease in transmitter released, and Dudel and Kuffler (210) ruled out experimentally that a postsynaptic conductance decrease or a block of conduction at the excitatory axons could participate in generating this special type of inhibition. The mechanism was postulated to be a

chemical transmission from the inhibitory to the excitatory ending on the basis of the special delay conditions necessary for the appearance of the inhibition (210).

Further analysis (197, 198) showed that the potentials recorded extracellularly near the excitatory axon terminal are decreased in amplitude or abolished when previous inhibitory nerve stimulation is timed to cause presynaptic inhibition. This effect was postulated to be due to a change in the excitatory nerve ending conductance to  $\text{Cl}^-$  or  $\text{K}^+$  (198, 200). Takeuchi and Takeuchi (806) demonstrated that removing  $\text{Cl}^-$  from the environment, replacing it with nonpermeant ions such as propionate, acetate, methylsulfate, or glycerosulfate, causes an increase in the quantal content of the EJP's, and almost no failures are recorded extracellularly from an individual excitatory junction. Moreover, when  $\text{Cl}^-$  has been replaced by impermeant anions and the inhibitory axon is stimulated 3 msec prior to the stimulation of the excitatory axon, no change in the amplitude of the EJP's is observed (806). This shows that the inhibitory transmitter also changes  $\text{Cl}^-$  permeability of the excitatory terminal (806).

GABA also mimics perfectly the effects of the inhibitory transmitter on the excitatory presynaptic endings (198, 200, 210, 805, 806). Perfusion of GABA on the junctions also causes a decrease in the quantal content of the EJP's recorded from an individual spot, without affecting the quantum size (805). This effect is attenuated or disappears when  $\text{Cl}^-$  is replaced by impermeant anions (805). This means that GABA, as the natural transmitter, increases the nerve terminal membrane permeability to  $\text{Cl}^-$  (806). GABA also causes a decrease in the extracellularly recorded excitatory nerve terminal spike but it does not affect the inhibitory nerve terminal at all (805). Electron-microscopic studies by Atwood and his collaborators (28, 29) have demonstrated the presence in crayfish muscle of axo-axonic synapses. These contacts probably underlie presynaptic inhibitory phenomena (see above).

Using the same methodology combining extra- and intracellular recording Dudel and Kuffler (209) analyzed the mechanism of facilitation of the EJP's by high-frequency stimulation in the dactyl abductor of the crayfish. This type of stimulation provokes an increase of the number of quanta released but does not alter the quantal size. These effects could be due to a depolarization of the nerve terminals (200).

Different drugs may act on crustacean presynaptic terminals.  $\beta$ -Guanidino-propionic acid, which acts as a competitive GABA antagonist on the postsynaptic membrane (252), may mimic the effects of GABA on the excitatory terminals (199, 201; see 203). 5-HT has been shown to increase the release of transmitter both in lobster and crayfish muscles (202, 342, 343, 708). Bacterial endotoxins may also facilitate the release of transmitter, also increasing the frequency of mEJP's (681). In contrast, strychnine seems to block the excitatory junctional transmission by affecting in an unknown way the presynaptic ending and without altering the postsynaptic membrane (680). Picrotoxin sensitizes the presynaptic ending to the action of phenethylamine, which in these conditions causes a backfiring effect (342, 343, 708). The action of several barbiturates, antiepileptics, analeptics, and neuroleptics has been analyzed by Iravani (425, 426). Barbiturates (Nembutal

and Trapanal) have effects on nerve conduction and block the effects of GABA on the postsynaptic membrane (426); some antiepileptics (parametadione, trimetadione, and phenacemide) depress the inhibitory transmitter release (426; see 203).

Some ionic changes may have effects on presynaptic endings. When  $Cs^{++}$  ions are substituted for  $K^+$ , the transmitter release by both excitatory and inhibitory endings appears increased (298). On the other hand, partial substitution of  $Mg^{++}$  or  $Mn$  for  $Na^+$  may block the EJP's without affecting the mEJP's (11). An increase of  $Ca^{++}$  may antagonize this effect (11).

F) RELEASE OF GABA FROM INHIBITORY NERVE ENDINGS. Otsuka et al. (671) demonstrated that electrical stimulation of inhibitory nerves innervating different exoskeletal muscles of the lobster *Homarus americanus* produces GABA release. The amount of GABA recovered from the perfusate after such stimulation is fairly proportional to the number of stimuli applied (671). Prior to performing these experiments in isolated legs of lobsters, the muscles are thoroughly washed with saline for 3–4 hr in order to reduce the spontaneous efflux of GABA to a minimum steady background level (671). In these conditions GABA is liberated by inhibitory nerve stimulation, whereas excitatory nerve stimulation does not affect the background spontaneous release. The amount of GABA released during the inhibitory nerve stimulation is rather variable. During eight 15-min periods of inhibitory nerve stimulation at frequencies of 5/sec, the average quantity of GABA released in excess above the background level amounts to  $1.5 \times 10^{-16}$  moles, whereas during five 15-min periods of inhibitory nerve stimulation at a frequency of 10/sec the average net release amounts to  $3.2 \times 10^{-10}$  moles (671; see 514).

Low- $Ca^{++}$  media reduce GABA release (671); after washing a muscle preparation for 4 hr with saline containing only 10% of the normal  $Ca^{++}$  content, the increase in GABA release above the background produced by stimulation is null when compared with a control preparation washed for the same period in normal saline (671). Junctional inhibitory potentials disappear when the muscles are perfused with low- $Ca^{++}$  medium. In none of these experiments does the absence of  $Ca^{++}$  impair the axon conduction (671). It is very probable that the amount of GABA collected after stimulation represents a small part of the actual amount of GABA released, because in parallel with this release a system of GABA uptake into the nerve endings is removing GABA from the synaptic clefts (514).

These experiments offer strong support, together with the other experimental evidence analyzed in this section, to the view that GABA is the transmitter released at inhibitory synapses of the lobster muscles.

G) UPTAKE OF GABA. From the subcellular distribution of the enzymes metabolizing GABA to succinylsemialdehyde, it is evident that these enzymes can hardly be involved in the inactivation of GABA when it would be released from nerve endings to the synaptic cleft. A GABA uptake system was found both in particulate fractions of mammalian brain (750) and in crayfish stretch receptor organs (767). Iversen and Kravitz (431) demonstrated that inhibitory nerve endings may also take up GABA from the extracellular space, the uptake system activated being similar to that found in the tissues already mentioned (see also 514).

When neuromuscular preparations of the isolated abdominal muscles of

*Homarus* are incubated in the presence of low concentrations of [<sup>3</sup>H]-GABA, the radioactive amino acid accumulates inside the tissue and its concentration rises linearly for 2 hrs (431). More than 95 % of the radioactivity in the tissue corresponds to intact [<sup>3</sup>H]-GABA, which may reach a value several times its concentration in the outside medium. This uptake mechanism is saturable, with an apparent  $K_m$  of  $5.8 \times 10^{-5}$  m (431, 514).

As with other systems transporting amino acids, the uptake system of [<sup>3</sup>H]-GABA at the neuromuscular junction depends on the Na<sup>+</sup> concentration in the extracellular space (431). Replacement of this cation by either Li<sup>+</sup> or choline results in an impairment of the uptake, whose intensity will depend on the amount of Na<sup>+</sup> replaced. Changes in the Ca<sup>++</sup> or K<sup>+</sup> concentration of the medium do not alter the uptake of radioactive GABA (431).

Among several compounds tested, only rather high concentrations ( $5 \times 10^{-4}$ – $10^{-3}$  m) of dimethylimipramine, chlorpromazine, and some close structural analogs such as guanidinoacetic acid,  $\beta$ -guanidinopropionic acid, and  $\beta$ -hydroxy-GABA were effective inhibitors of [<sup>3</sup>H]-GABA uptake (431). Orkand and Kravitz (662), using electron-microscope radioautography, have recently tried to localize the site where radioactive GABA is taken up in lobster muscles. Only 30 % of the GABA taken up may be bound to protein by fixation with glutaraldehyde either alone or mixed with acroleine or paraformaldehyde (662). Surprisingly, the label appears to accumulate over Schwann cells and connective tissue cells, sometimes on muscle fibers but very rarely over nerve terminals. This distribution does not seem to arise from displacement artifacts, but since endings are not so numerous as to appear in each section, it is not sure that those examined were inhibitory endings (662). It is difficult to ascertain whether this observation reveals an actual role of Schwann cells or connective cells in GABA uptake (662). In this sense these observations are at variance with data from vertebrate brain where uptake of labeled GABA has been found localized in the synaptosomal fraction (635). On the other hand, a relation between the capacity of GABA uptake and the amount of inhibitory innervation has been observed when comparing [<sup>14</sup>C]-GABA uptake by different crustacean muscles with different inhibitory innervation (623). GABA uptake was studied in the lobster stomach muscles, which do not receive any inhibitory inputs; in the fast central fibers from the extensor muscle of the carpopodite of *Pachygrapsus*, which presumably receive only scanty inhibitory fibers; in the slow proximal fibers of the latter muscle, which receive only one inhibitory axon; and in the opener muscle fibers of the claw of *Pachygrapsus*, which receive two inhibitory axons (623). When all these muscles are incubated in selected standard conditions with [<sup>14</sup>C]-GABA ( $10^{-4}$  m) for 45-min periods, a condition previously found to be the most suitable, a general correlation between the type of innervation of the muscle and the net GABA uptake is found. The net GABA uptake is very low in the stomach muscle of *Homarus* devoid of inhibitory input and appears to be maximal (i.e. 20 times greater) in the *Pachygrapsus* opener muscle fibers, each of which receives two inhibitory fibers (623).

Summarizing the evidence presented here, it must be said that the transmitter function of GABA in inhibitory neuromuscular transmission of *Crustacea* has been proven.

4) *Excitation and glutamate.* In crustaceans ACh does not affect the muscle fibers of nor is it detectable in the motor nerve fibers (41, 42, 269, 458).

At the end of the 1950's, as a result of work on GABA and inhibition, a series of dicarboxylic amino acids capable of causing contraction in crustacean muscle fibers attracted the interest of physiologists (715, 887, 888). The most active of these amino acids was L-glutamic acid (715, 888), which when perfused on claw opener muscle fibers of the crayfish at concentrations of  $2 \times 10^{-5}$  M may cause a slight and definite facilitation of the muscle contraction produced by stimulation of excitatory axons. At concentrations of  $10^{-4}$  M it may elicit a contracture. Both effects on muscles are transient and easily reversible after removal of the amino acid. Higher concentrations produce more sustaining contractures and block the effects of nerve stimulation (715, 888). D-Glutamic acid was ineffective on muscle fibers even at concentrations of  $10^{-3}$  M. The same observations were confirmed in a different way: glutamic acid was identified by column and paper chromatography of brain extracts as the more active compound in producing crustacean muscle contraction (886-888).

Intracellular recordings show that muscle contraction induced by glutamate is always associated with depolarization (801, 802, 886); the effects on membrane potential are transient, as are those on contraction. The effectiveness of glutamate is decreased by either inhibitory nerve stimulation or mixing with GABA (888).

All these data pointed to the idea that glutamate may be involved in the physiological excitation of crustacean muscle fibers. However, some objections were raised because of the wide distribution and high concentrations of glutamate in muscle and other tissues and the high threshold of the muscle response to glutamate, contrary to the usual high sensitivity of the synaptic structures to a transmitter (888). An alternative hypothesis was proposed (887) suggesting that the transmitter could be an analog of glutamate. Numerous biochemical and physiological studies later brought forth more arguments in favor of an excitatory transmitter role for glutamate (see 520).

A) **BIOCHEMICAL DATA.** Glutamic acid is the main excitatory substance found in aqueous extracts of lobster (*Homarus americanus*) CNS (205, 515, 516, 518, 520). Glutamate has been identified in this nervous system by paper chromatography with different solvents: butanol-acetic acid, butanol-pyridine water and phenol water (205). Glutamate is present at the same concentrations in both excitatory and inhibitory axons isolated from the lobster leg, its axoplasm concentration in each amounting 0.003 mM (515, 516, 518). A high glutamate content was also found in leg nerves of *Carcinus* (561). Single-cell analysis shows that glutamate is also present in both motor and muscle inhibitory cells isolated after physiological identification from lobster ganglia (672). The glutamate concentrations in two identified inhibitory neurons and in two identified excitatory neurons appear rather similar: 14-20 mM in the inhibitory neurons and about 18 mM in the excitatory neurons (672). This ubiquity of glutamate could be considered an indication against its participation as an excitatory transmitter; however, it must be kept in mind that glutamate is also involved in the Krebs cycle and furthermore, especially in the crustacean nervous system, it is the precursor of GABA.

B) **GLUTAMATE RECEPTORS.** Perfusion experiments do not yield much informa-

tion about the localization of glutamate receptors or the relationship of glutamate with the receptors to the natural excitatory transmitter. This problem was carefully analyzed by Takeuchi and Takeuchi (801, 802), and it was found that glutamic acid has a localized action on the junctional regions of the crustacean muscle fiber (675, 676, 801, 802). Iontophoretic application of glutamate along the surface of the abductor muscle of the dactylopodite of the crayfish walking leg detects only a series of very circumscribed sensitive spots (801, 802). The response of the muscle fibers to glutamate appears as transient depolarizations, their time course and amplitude depending critically on the position of the glutamate micro-pipette (520, 675, 676, 801, 802). By applying a solution of the diffusion equation (188) Takeuchi and Takeuchi (802) calculated that a 0.045-mm concentration of glutamate might reach the receptor when a depolarization of 3 mv is caused by the iontophoretic application from a micropipette containing a 1-mm solution of glutamate, located at a distance about 9  $\mu$  from the receptors. The contraction threshold is 0.035 mm (887). Lobster muscle appears to be less sensitive than crayfish muscle (520, 675).

By combining intracellular and extracellular recording it appears that the areas of glutamate sensitivity coincide with the regions where the EJP's are recorded extracellularly. Takeuchi and Takeuchi (812) could obtain with minimum currents glutamate responses of the same amplitude as the EJP's, but a difference still appeared between the time course of the two responses: the time to peak of the glutamate potential is twice as long as the time to peak of the EJP. In these experiments the calculated distance from the pipette to the receptors was 4  $\mu$  and the concentration of glutamate causing the response was  $5 \times 10^{-17}$  m. A possible objection is that glutamate could be acting indirectly by releasing transmitter from nerve endings (802). A method to overcome this objection by degenerating the nerves innervating the muscles has been attempted (802), but unfortunately transected crustacean nerves degenerate only after very long periods and regeneration may be achieved by fusion of the remaining stumps (394, 395).

The receptors to L-glutamate are highly specific; they are neither activated nor blocked by D-glutamate (802). Repetitive application of glutamate iontophoretic pulses to the sensitive spots resulted in potentiation of the first response after a test response and desensitization of the successive ones (802). This finding explains the transient effects of glutamate perfused on muscle (715, 888).

Crossed desensitization between glutamate and the natural transmitter could be a good test to show their identity. In fact Takeuchi and Takeuchi (802) observed that prolonged application of glutamate caused a diminution in the amplitude of the EJP recorded intracellularly and extracellularly from a spot on the same muscle fiber. This effect of glutamate persists after switching off the iontophoretic current and could be due to desensitization effects and not to the action of the amino acid on the membrane conductance of the fiber. An alternative explanation could be a presynaptic effect of glutamate depolarizing nerve endings (see 277). No attempts have been made to desensitize glutamate receptors with a barrage of EJP's; this may be difficult to observe because of the multiple innervation of the muscle fiber.

C) IONIC MECHANISM OF EJP AND GLUTAMATE RESPONSES. The amplitude of the EJP's and the glutamate potentials decrease more or less linearly when the muscle fiber membrane is depolarized from  $-60$  to  $-20$  mv (675, 676, 813). When the fiber is hyperpolarized, the EJP and the glutamate responses also decrease in amplitude. These changes are due to a rectification in the fiber membrane (675, 676, 813) due to an increased  $\text{Cl}^-$  permeability (676). The glutamate responses may be made larger with hyperpolarization when the intracellular  $\text{Cl}^-$  is reduced by washing with a  $\text{Cl}^-$ -free propionate medium, which suppresses the rectification (676). High doses of picrotoxin ( $10^{-3}$  M) may also abolish the non-linear behavior of the electrically excitable membrane (676). Under these conditions the extrapolation of the straight line relating  $E_m$  and the amplitude of the glutamate response shows that the glutamate potential becomes null around  $-18$  mv (676).

A similar value was found (802) for the saturation level of the glutamate dose-response curve (mv depolarization vs. glutamate concentration). In some preliminary experiments using the voltage-clamp technique (802) the reversal level of the glutamate currents appear to be around the zero potential level when the muscle is bathed in a normal saline.

Generally there are some difficulties in measuring  $E_{\text{EJP}}$  because of the membrane rectification and the multiple terminal innervation of the individual fibers (802, 813). To overcome these difficulties extracellular EJP's were recorded from individual synaptic spots and averaged, because of their low quantal content (813). In these conditions an  $E_{\text{EJP}}$  of about  $-4$  mv may be observed, which is different from the  $E_{\text{EJP}}$  values of  $-20$  mv and  $-12$  mv calculated by extrapolation in the same lobster fibers (813). A similar difference appears between the observed reversal potentials of glutamate responses of about  $-6$  mv and the calculated values of  $-12$  mv. In some cases positive values of  $E_{\text{EJP}}$  are observed, which are difficult to explain (813).

In spite of these still unexplained differences, both the  $E_{\text{EJP}}$  and  $E_{\text{glutamate}}$  appear to have a similar ionic generating mechanism consisting of an increase in  $\text{Na}^+$  conductance probably coupled with an increase in  $\text{K}^+$  conductance (206, 676, 709). Replacement of the  $\text{Na}^+$  in the saline by  $\text{Li}^+$  slowly reduces the amplitude of the EJP and after long exposure (more than 2 hr) finally abolishes it (676). Glutamate potentials are completely abolished after 25 min of exposure to a  $\text{Na}^+$ -free  $\text{Li}^+$  solution. This effect of  $\text{Na}^+$  removal on the EJP cannot be due to an action on the axon spike, since this is not affected by substitution of  $\text{Li}^+$  for  $\text{Na}^+$  (248). It is difficult to determine if there is not an effect of such long exposure to  $\text{Li}^+$  on the  $\text{Na}^+/\text{K}^+$  pump, causing both external  $\text{K}^+$  accumulation and changes in the  $\text{Na}^+$  gradient.

The replacement of  $\text{Na}^+$  by tris Cl acts much more rapidly on the glutamate potential, abolishing it in 8 min. Replacement of a part of the tris Cl by  $\text{CaCl}_2$  does not seem to greatly change the effect of  $\text{Na}^+$  removal on the EJP's (676). In the absence of  $\text{Na}^+$ , glutamate does not seem to affect the membrane potential even if the resting potential is shifted away from  $E_K$ . It seems then that, even if  $\text{K}^+$  is involved in the genesis of the glutamate potentials, when the inward flux

of  $\text{Na}^+$  is abolished the increase in  $\text{K}^+$  permeability may also be abolished (676). Neither the glutamate response nor the EJP is at all affected by poisons like tetrodotoxin and saxitoxin (674).

D) GLUTAMATE VERSUS ASPARTATE. Chemical studies on the extracts of lobster nerve showed that glutamate and aspartate together make up most of the excitatory activity of the extract (520). However, L-aspartate is at least 400 times less effective than L-glutamate on *Cambarus* muscles (812).

In the lobster it is possible to obtain by perfusion of appropriate doses similar depolarizations with aspartate and glutamate [Slater et al. (520)]. The aspartate required must be 2–4 times more concentrated than glutamate. Aspartate may potentiate the effect of glutamate even in doses apparently not active by themselves (520). Therefore, it would appear that glutamate is much more effective than aspartate.

E) RELEASE OF GLUTAMATE FROM NEUROMUSCULAR PREPARATIONS. Recently Slater et al. (520) observed a small additional release above a large background efflux of glutamate when excitatory nerves to the opener muscle of the dactyl of lobster were stimulated during perfusion of the preparation with lobster physiological saline (5–10 C). In these conditions a very high resting efflux of glutamate ( $3.5-8 \times 10^{-10}$  moles/25 min) is observed, which cannot be prevented. This is a small fraction of the glutamate present in the nerve but it is similar to the quantity of GABA that may be released from the inhibitory nerve by stimulation periods of 25 min (671) or to the calculated quantity of excitatory transmitter that would be released in 1 hr by the motoneuron when it discharges with a frequency of 20/sec (73). Due to this large background, in 39 experiments a statistically significant increase of only 10% of the glutamate release was obtained by nerve stimulation. Experimental difficulties impair experiments by changing  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  to demonstrate whether this released glutamate comes from nerve endings. Exogenous radioactive glutamate is taken up by neuromuscular preparations (520). These preparations do not release labeled glutamate when the nerves are stimulated at 5/sec, but when the muscles are made to contract the preparation releases a quantity 5–6% above the background. This release may be abolished by increasing 3 times the  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  concentrations of the medium, a procedure that also blocks the EJP's.

These data on glutamate release are not as convincing as the evidence on GABA release in the same preparations.

F) INACTIVATION OF GLUTAMATE. It is difficult to find clear evidence for a possible enzymatic inactivation of glutamate, if it is indeed released as a transmitter by excitatory presynaptic endings. Enzymes known to convert glutamate to physiologically inactive compounds (glutamine and  $\alpha$ -ketoglutarate) are not able to dispose of glutamate at the synaptic cleft because of their localization and the necessity of some cofactors.

Iversen and Kravitz (431) have described a transport system for glutamate in the lobster nerve-muscle preparation. This uptake system is saturable, requires the presence of  $\text{Na}^+$  in the extracellular medium, and seems to be specific for glutamate (see similar results in sect. vB). The absence of specific blocking agents

for this transport hinders further analysis of its participation in the handling of the natural excitatory transmitter.

Nerve stimulation increases the uptake of cytochemically demonstrable particles of horseradish peroxidase in lobster muscle endings. Of the vesicles 20–30% have been found to contain peroxidase (391). It is difficult to know yet whether or not this process is related to transmitter uptake or if it is a pinocytic process related to the incorporation of a different material from the synaptic cleft.

G) GLUTAMATE VS. EXCITATORY TRANSMITTER. Kravitz et al. (520) recently pointed out that in crayfish and lobster "glutamate remains not only the leading candidate for the excitatory transmitter compound but also the only one." The following points favor this hypothesis: 1) it is the main excitatory compound in extracts of whole nervous systems, peripheral nerve, and in isolated central nervous perikarya of *Crustacea*; 2) the receptors to glutamate are localized in the same spots as the receptors of the natural transmitter and their sensitivity are not very different; 3) both glutamate and the natural transmitter produce the same change in membrane permeability; 4) in spite of an enormous background leakage, it is possible to detect a small significant release of glutamate by nerve stimulation; 5) a transport system is able to take up glutamate from the extracellular space, thus providing a possible mechanism of inactivation.

Now let us examine the objections to a transmitter function for glutamate. The two most important are its ubiquity and the presence of a high content of glutamate in the hemolymph. As Kravitz et al. (520) point out: "there is no known reason why a compound cannot serve an important metabolic role as well as functioning as a transmitter compound. All that it is required is that the cells regulate the level of the compound so that it can serve adequately both purposes." With respect to the problem of the glutamate level in hemolymph, Florey (266) has emphasized the difficulty for a substance to be a transmitter when it is in the hemolymph at concentrations some 100 times higher than the dose required to produce physiological effects when perfused on the muscle. Slater et al. (520) thoroughly analyzed this problem and found a very variable content of glutamate and aspartate in the hemolymph of the lobster: approximately  $0.7 \times 10^{-4}$  M of glutamate and  $4 \times 10^{-4}$  M of aspartate. For some reason the change from a physiological saline environment to hemolymph produces hyperpolarization of the muscle fiber. When glutamate is added to the hemolymph in concentrations of  $5 \times 10^{-4}$  M and this hemolymph is perfused to the preparation, a depolarization of 10 mv and a 50% reduction of the EJP's are produced. These experiments show that the normal level of glutamate in hemolymph may not affect the muscle junction. Identical findings in insect muscle have been reported by Usherwood (see 874). Whether glutamate exists in an inactive form in the hemolymph, where it is inactivated by an unknown substance, remains a matter of pure speculation.

### C. Stretch Receptor Organs

Inhibitory peripheral nerves of crustaceans also innervate sensory peripheral neurons forming part of what Alexandrowicz (3–5) called the "muscle receptor

organs." The evidence about inhibitory transmission to this neuron complements the previous section on neuromuscular junctions (sect. ivB). Moreover, historically the elegant work by Kuffler and his colleagues (see 525) contributed to a larger view of neuronal inhibition and gave further impulse to the hypothesis that GABA could be an inhibitory transmitter.

1) *Structural background.* Alexandrowicz (3, 4) described in the dorsal region of the abdomen and thorax of the lobsters *Homarus* and *Palinurus* muscle receptor organs composed of different elements: *a*) some slender muscle fibers; *b*) a sensory neuron with its dendrites in close contact with the muscle fibers; *c*) an innervation consisting of motor nerves and two "accessory" nerves, one much thinner than the other; and *d*) the neuron axon running to the CNS. The two accessory nerves were found to be inhibitory both in crayfish and in lobster (106, 239). Alexandrowicz (3, 4) postulated that the muscle receptor organs could actually be stretch receptor organs (SRO). They have been found in every species of *Crustacea* analyzed to date for this purpose (272, 692, 932; see review in 5).

Wiersma et al. (932) first demonstrated the sensory nature of SRO by applying tension to the muscular ends of the structure and recording repetitive discharges from the afferent axon. There are two types of SRO: one "slow adapting," able to sustain a continuous discharge for long periods of stretch, and the other "fast adapting," showing a high threshold to stimulation but adapting to an extreme stretch in less than 1 min (239, 262, 523, 932).

The ultrastructural studies have mainly concentrated on clarifying the relationships between dendrites and muscles and especially on describing the endings of the inhibitory axons. In comparing the submicroscopic morphology of excitatory and inhibitory nerve endings, the SRO is rather exceptional: the neuron only receives inhibitory afferents (527, 863), whereas the muscle fibers, like other exoskeletal muscle, receive both excitatory motor and inhibitory inputs (5, 254, 510, 631).

The fine structure of fast- and slow-adapting neurons is similar in lobster and crayfish (81, 631). The cell body is rich in endoplasmic reticulum, free ribosomes, and elongated mitochondria. The axon hillock shows a particular concentration of these organelles. The axon is devoid of ribosomes and poor in mitochondria. The dendrites contain microtubules and elongated mitochondria. The axon and the cell body are ensheathed by many alternate layers of Schwann cells and connective tissue. Complicated infoldings of Schwann cells are observed around the perikaryon. The dendrites are enveloped only by a thin layer of Schwann cell processes and appear embedded in dense connective tissue (81, 631, 685).

Synaptic contacts are observed mainly on the dendrites (631, 685, 863). The axon endings show localized presynaptic densities with vesicles attached. A synaptic cleft of 200 Å separates both membranes. The axon endings of glutaraldehyde-fixed SRO are full of flattened or elongated vesicles of 200–300 Å in the short axis and 400–600 Å in the long axis (631, 863). Since these terminals correspond to endings of inhibitory nerves [the same features have been found in presynaptic endings of axo-axonic synapses at crustacean neuromuscular junctions (see sect. ivB)], Uchizono (862–864) has generalized the idea that inhibitory and excitatory

endings in vertebrates and invertebrates can be distinguished by the shape of the vesicles they contain: the inhibitory endings contain flat vesicles and the excitatory ones contain round vesicles. This is a very risky extrapolation, since the inhibitory or excitatory character of a transmitter depends on the ionic permeability changes that it elicits on the postsynaptic membrane. It may be remembered, for instance, that ACh may excite skeletal muscle fibers of vertebrates and inhibit the cardiac muscle fibers (for a similar situation in molluscan CNS see sect. viA). Therefore, it may be supposed that the shape of the vesicles may be related to a particular transmitter, very probably GABA in the SRO.

The neuromuscular endings on the muscle fibers of the SRO are of two types: one resembles the axodendritic endings on the neuron and contains flattened vesicles (510, 631); the other (510, 631, 863) contains clear spherical vesicles 400–900 Å in diameter. It is likely that the latter correspond to excitatory motor endings (see also 28, 30).

2) *Properties of stretch receptor organs.* The experiments of Eyzaguirre and Kuffler (239, 240, 527) have given us a rather complete picture of the microphysiology of the SRO, which can only be briefly summarized here. Details can be found in the review articles by Kuffler (524, 525), Eyzaguirre (238), and Edwards (217).

When impaled with microelectrodes, isolated stretch receptor neurons show resting potentials of from –70 to –80 mv. When the receptor muscle is stretched the neurons become depolarized and may fire action potentials. The two types of receptors have different threshold levels; a depolarization of 8–12 mv is necessary to discharge the slow SRO, whereas the fast one only fires when the neuron is depolarized by 18–22 mv (239). In the slow SRO the discharge is maintained as long as the stretch, whereas in the fast SRO the cell discharges briefly and then comes back to the resting level even if the stretch is maintained, only a new increase in stretch causing new spike firing (239).

The depolarization is probably generated by dendrite deformation (239, 523). It is then similar to the generator potential described in other receptors. The depolarization spreads electrotonically from the dendrites to the cell body and axon. Generator potentials are then graded and decremental. Overstretch may cause a block of the spike discharge (239). In the slow-adapting SRO there is good evidence that the axon hillock region has a lower threshold than the neuronal somata and that afferent spikes are generated there, invading the somata secondarily (220). The membrane potential of the stretch receptor neuron varies linearly with  $[K]_o$  but the change in  $E_m$  for a 10-fold variation in  $[K]_o$  is only 35 mv (222). Therefore, other ionic mechanisms must also intervene in the regulation of  $E_m$ . Increase in  $[K]_o$  or removal of  $\text{Ca}^{++}$  may cause a drop in membrane resistance (222).  $\text{Cl}^-$  permeability does not seem to contribute to  $E_m$ , since replacement of the entire  $[\text{Cl}]_o$  by glutamate does not alter  $E_m$  (347). It is likely that an electrogenic  $\text{Na}^+-\text{K}^+$  active transport mechanism could participate in the generation of  $E_m$ . Nakajima and Takahashi (632) have recently shown that tetanic stimulation of crayfish stretch neurons activates an electrogenic  $\text{Na}^+-\text{K}^+$  pump, causing a long-lasting posttetanic hyperpolarization. Moreover, replacement

of the whole  $[Na]_o$  by  $Li^+$  would cause neuron depolarization, probably due in part to the inactivation of an electrogenic pump (651).

The action potentials of the stretch receptor neurons are blocked by replacement of  $Na^+$  by choline or sucrose (222). Replacement of  $Na^+$  by  $Li^+$  does not alter the generator potential as happens in other receptor structures but it blocks spike generation (651). Tetrodotoxin blocks the neuronal action potentials (432).

3) *Inhibition and GABA.* Stimulation of "accessory" nerves in both crayfish and lobster stops the firing of action potentials or impedes their discharge (106, 527). This inhibition of spike discharge lasts as long as the nerves are stimulated and despite maintenance of a sustained stretch in both types of SRO.

The polarity of the inhibitory potentials recorded intracellularly after nerve stimulation depends on the membrane potential (527). When the muscle cells are relaxed each stimulation causes a depolarizing synaptic potential. As the muscle is stretched the membrane becomes depolarized, and the synaptic potentials reverse and become hyperpolarizing, the amplitudes of these IPSP's being dependent on the degree of membrane depolarization caused by the stretch (527). Thus the polarity and the amplitude of the IPSP's depend on the  $E_m$  and they always tend to displace  $E_m$  to the level of  $E_{IPSP}$ . The IPSP's may reach amplitudes of about 15 mv and durations of 30–35 msec, the time to peak being only 2 msec. They may show summation as well as aftereffects in the form of depressions or facilitations after prolonged repetitive inhibitory stimulation (527; see 238). The IPSP's may block the discharge of action potentials when evoked in an appropriate time sequence, and they may also affect the afterpotentials of antidromic impulses (527). The reversal potential of the IPSP is close to  $E_m$  (5–8 mv) when it is measured either by changing  $E_m$  by stretch depolarization or by the current-clamp method (347, 527). A reversal potential of –70 mv was measured in a neuron that had an  $E_m$  of –75 mv (527). Miniature IPSP's were found to reverse at –66 mv (432).

A) **IONIC BASIS OF IPSP's.**  $Cl^-$  ions seem to be the main ions involved in the membrane permeability change generating the synaptic current. Injected into the cell from intracellular KCl-filled micropipettes,  $Cl^-$  reverses the polarity of the IPSP's (347, 527) or the mIPSP's (432). The depolarizing IPSP's thus obtained are capable of firing spikes (347). When the entire  $Cl^-$  content of the saline bathing a slow SRO is replaced by glutamate the reversal of the IPSP's appears at the membrane potential levels at which they were previously hyperpolarizing (347, 673). Replacement of the entire  $[Cl]_o$  of the environment by propionate also causes a reversal of the polarity of mIPSP's (432). It is more difficult to evaluate the participation of a  $K^+$  permeability change in the genesis of the IPSP, as suggested by many workers (218, 347, 432).

The main arguments in favor of such an intervention of  $K^+$  permeability in the genesis of the IPSP are: 1) complete removal of  $K^+$  from the environment causes hyperpolarization of the neuron, but instead of the expected reversal of the IPSP appearing when  $E_m$  increases the IPSP's persist as hyperpolarizing deflections and increase in amplitude (218); 2) an increase in  $[K]_o$  in the environment of a neuron where normally the mIPSP's were depolarizing then depolarizes

the neuron, but instead of the reversal of the mIPSP's the depolarization was expected to cause the mIPSP's continue to depolarize and even increase in amplitude (432). All these arguments are valid if it is ensured that by changing [K], no changes in [Cl]<sub>i</sub> have been introduced. That would be an important point to establish, because this is the only case in invertebrates of an inhibitory transmitter apparently acting on only one receptor that could change the membrane permeability to both an anion and a cation. Osawa and Tsuda (673) have recently claimed that stretch receptor neuron inhibition in crayfish alters only Cl<sup>-</sup> membrane permeability.

**B) MINIATURE ACTIVITY.** Miniature inhibitory activity may be recorded in crayfish stretch receptors (432). They appear depolarizing when KCl microelectrodes are used but are almost always hyperpolarizing when K<sub>2</sub>SO<sub>4</sub> pipettes are used. This miniature activity is the same in both slow and fast SRO. Miniature IPSP may reach 1 mv in amplitude with durations that show a great variation—between 5 and 130 msec. This suggests a wide spread of inhibitory endings on the neuron surface (432). When the intervals between mIPSP's are plotted over a theoretical curve describing complete randomness of frequency, a close fit between both curves is observed (432). An increase in K<sub>o</sub> does not seem to alter the frequency of discharge of the mIPSP's. It is difficult to calculate the quantal number in the SRO neurons because both  $E_{IPSP}$  and  $E_m$  are very close. However, Iwasaki and Florey (432) calculate that at least 80 quanta could be expected to be released by an impulse from inhibitory terminals on the stretch receptor neuron. This number is similar to that found by Dudel and Kuffler (208) in the inhibitory neuromuscular junctions of the crayfish.

**C) ACTION OF GABA.** The stretch receptor neuron was the preparation preferentially used for a long time as a bioassay tool for the screening of inhibitory substances. Florey (258, 259) extracted from mammalian brain an inhibitory compound, factor I, that blocked the responses of the SRO to stretch. The properties of factor I have been commented on in detail previously (sect. IVB). Let us remember here that Bazemore et al. (53) showed that the inhibitory activity of brain extracts containing factor I was principally due to the presence of GABA. Eliot et al. (231) showed that lobster nervous system extracts could also inhibit the SRO neuron and also probably contained GABA. This line of research was successfully pursued by Kuffler, Kravitz, and their colleagues, as reported in detail in section IVB (see also 512, 513).

Early in these studies GABA was shown to be the most potent inhibitory substance on SRO neurons (219, 526). Concentrations about  $10^{-5}$  M are able to stop the discharge of the neuron and to hold the membrane potential under the threshold of spike discharge (526). Lower concentrations slow the discharge. Completely relaxed SRO's are not apparently affected by GABA. On the other hand, during GABA action, stretch is not more effective in eliciting depolarization and discharge of the SRO neuron (219, 526).

GABA produces increased membrane conductance of the SRO neuron (347, 432, 526). The action of both GABA and the IPSP's tends to the same equilibrium potential when measured on the same neuron (347, 432, 526, 673). Thus

no IPSP's are seen when the neuron is bathed in  $10^{-5}$  M GABA. GABA responses are also mainly due to a change in  $\text{Cl}^-$  membrane permeability (347, 432, 673). The alleged participation of  $\text{K}^+$  permeability in GABA action (347, 526) may be subject to the same criticisms discussed above for the IPSP's and deserves further investigation.

No work concerning specific localization of GABA receptors on the surface of the SRO neuron is available but an interesting finding of Kuffler and Edwards (526) is that the median receptor of the 7th thoracic segment in *Homarus*, which according to Alexandrowicz (4) is devoid of inhibitory inputs, is also sensitive to GABA; no IPSP could be recorded from these SRO's, but GABA was able to block both the stretch discharges and the antidromic firing. This shows that GABA receptors may also be present on the neuron membrane outside the subsynaptic regions (526).

The structure-activity relationship of different inhibitory amino acids was thoroughly analyzed by Edwards and Kuffler (219), who found that the  $\text{NH}_2$  and the  $\text{COOH}$  groups were essential for the inhibitory action, which was optimal when, as in the case of GABA, these end groups were separated by three carbon atoms. The inhibitory action of the compounds is less effective when the chain is either lengthened or shortened. Some guanido acids, like guanidoacetic acid, are as powerfully an inhibitor as GABA itself (219). Imidazolylacetic acid and taurine may show analogous inhibitory potency (598). Glutamate was also found to inhibit the impulse activity (328, 329).

As in the neuromuscular junctions of arthropods both the GABA responses and the IPSP's are blocked by picrotoxin (432). Picrotoxin was shown in the early studies to block the effects of factor I on SRO neurons (232). Picrotoxin itself has a depolarizing action on the neuron and causes a slight increase in membrane resistance. These findings have been recently interpreted as due to the blocking of miniature inhibitory activity that normally would maintain a certain level of increased membrane conductance (432).

No studies are yet available on the release of GABA in stimulated stretch receptor preparations, but the data obtained in muscular inhibitory nerve endings of lobster (see sect. IVB) may be applicable to SRO inhibition.

Kuffler and Edwards (526) observed that when GABA application was prolonged the effects on membrane potential subsided, which was interpreted as not being due to a prolongation of the effect on the membrane resistance nor to a desensitization, since stirring of the solution around the neuron reactivated the effects of GABA application (526). GABA did not appear to desensitize the receptors, and Kuffler and Edwards (526) postulated that some uptake mechanisms could be responsible for the GABA inactivation. An uptake mechanism has been demonstrated in peripheral inhibitory endings innervating exoskeletal muscle in *Homarus* (see sect. IVB). On the other hand, Sisken and Roberts (767) demonstrated that [ $^3\text{H}$ ]-GABA was taken up by isolated SRO neurons, using an autoradiographic technique. This binding takes place even at low temperatures (0–4°C) and depends on  $\text{Na}^+$  (767). The binding sites were thought to be located in axodendritic synapses (see 719) but the lack of resolution of the technique makes this localization unreliable.

D) OTHER PHARMACOLOGICAL STUDIES. The possible existence of a cholinergic mechanism involved in the function of SRO was repeatedly explored but only negative results were obtained. Cholinesterase histochemically detected with the Koelle method was abundant in the sensory neuron, with more in the fast than in the slow type (585). However, no indications about ACh transferase activity were obtained until very recently, when Hildebrand et al. (380, see 44a) demonstrated with a high-voltage electrophoresis method that single SRO neurons could synthesize both GABA and ACh. Since there is solid proof that GABA is synthesized by crustacean inhibitory nerve endings (513; see sect. ivB), it is likely that ACh is synthesized by the SRO neuronal somata itself, which thus will be cholinergic (380). Nerves, probably sensory, running in crustacean ganglion roots also have been shown to be cholinergic (269, 380).

Pharmacological interest in the ACh actions on the SRO was raised by the finding of Wiersma et al. (932) that it initiates rhythmic responses in slow-adapting receptors, these oscillations being potentiated by eserine and blocked by atropine. The same observations were made by other workers (263, 599), and it was found that the SRO neurons were excited by both muscarinic-type and nicotinic-type agonists (599). The action of these cholinergic agonists on the neurons may be blocked either by atropine, hexamethonium, or  $\beta$ -erythroidine (599). These effects could have been considered interesting at the time SRO neurons were thought to receive an excitatory input, but since this possibility has been discarded these findings have today only a purely pharmacological interest.

The same situation seems to be applicable to the effects of DA, which has been found to have a blocking action on the impulse discharge of SRO neurons in some species of crayfish [*Pacifastacus lemnisculus* and *Procambarus clarkii* (597, 598)]. In other species (*Orconectes propinquus*) this drug has no activity (598). The interest in DA was raised mainly by the fact that it was found to be a component of factor I extracted from mammalian brain (see sect. ivB).

From the evidence presented and the complementing studies in neuromuscular junctions (sect. ivB) it appears that GABA is the transmitter of inhibition to the SRO neurons of *Crustacea*.

## V. INSECTS

### A. Central Nervous System

For the insect CNS, as in other arthropods and other phyla, there is a predominance of studies on a very restricted number of species. Recent work has indicated that ACh and GABA may mediate central synaptic activities. A rather large number of reviews have been devoted in the last decades to the problems of function and pharmacology of the insect CNS. The more recent are those of Treherne (840), Boistel (82), and Pitman (694).

1) *Structural background.* The general anatomy of the different parts of the insect CNS is detailed in Bullock and Horridge's treatise (105). Generally the central ganglia appear surrounded by collagen forming the superficial "neural"

lamellae (779) that ensheathe a layer of flat cells, the perineurium. The fine structure and the functional implication of these envelopes are discussed later.

Under these sheaths there is a monolayered or multilayered array of monopolar pear-shaped neurons (108). These cells send their axons to the core of the ganglia, where they branch and contribute to the formation of a very complicated, partly submicroscopic, neuropile. Glial cells form a very complex structure around the perikarya; thin glial cell processes are there separated by irregular spaces [the "lacunar system" (see 777, 937)]. In several species of insects, Trujillo Cenoz (848) recognized three types of glial cells: capsular, neuron satellites, and fiber satellites (see also 545, 776).

No axosomatic synapses have been described in insects. Somatosomatic contacts with the characteristics of gap junctions are observed in *Formica* (549). They resemble the structures corresponding to electrotropic interneuronal synapses described in other phyla (see 58). In the early ultrastructural studies (367, 847) all the synapses observed were of the axo-axonic type, and this finding has been confirmed again and again in different CNS structures of various species such as the ganglia and brain of *Periplaneta americana* (12, 367, 573, 777), nerve cord and brain of different species of *Formicidae* (545-547, 785, 849), in ganglia of *Calopteryx splendens* (96), in the CNS of *Pholus labrusco* (847), and in the CNS of blowfly larvae (663).

In all these descriptions there is general agreement about the complexity of the neuropile and the observation of two types of axo-axonic contacts: *a) en passant* or longitudinal (847, 848); and *b) terminal*, constituted by axon end knobs contacting one or multiple axonic branches (see 775, 777).

Varicosities, full of microvesicles that appear along the axonal trajectories, may have a presynaptic function (575) but, as in terminal endings, the localization of synaptic regions can only be ascertained by the presence of clumps of synaptic vesicles attached to the inner face of a presynaptic membrane (773). Methods such as the zinc-iodide technique or PTA block staining may facilitate a more precise localization of the synaptic regions (546).

Complex synaptic structures are also found, especially in insect brains, like the so-called glomeruli and "diads." The glomeruli (785, 849) are rather different from the vertebrate glomeruli and consist of a large knoblike nerve ending that contacts more than a dozen axonic fine branches. These regions of contact show enlarged extracellular clefts associated with membrane densities on each side and with agglomerations of synaptic vesicles attached to the internal surface of one of the membranes. In the brain of the ants *Formica* and *Camponotus*, some regions of the pre- and postsynaptic axons participating in the glomerulus also seem linked by tight junctions (849). It is difficult to know if these latter junctions are artificial. The diads (547) resemble the disposition of elements composing some vertebrate retinal synapses: the presynaptic element has a T-like form and becomes actually embedded in a deep invagination of the postsynaptic fibers. Discrete synaptic regions appear between the elements. "Serial" synapses (synapses on synapses) have also been observed in blowfly brain (663).

Insect central synaptic endings generally contain clear vesicles 300-500 Å

(363, 414, 777). Sometimes these elements are mixed with more voluminous granulated vesicles (547, 849). Large granulated dense-core vesicles 600–1100 Å with the aspect of elementary neurosecretory granules are found in many neuropiles (see for instance 133, 414, 573, 777). Other contents of synaptic endings are similar to those described in other phyla, such as abundant mitochondria and glycogen particles.

2) *Properties of neurons.* Insect central neurons and giant axons may be successfully impaled with intracellular micropipettes. Giant axons of omnivorous insects show rather high resting potentials of around  $-70$  mv (83, 952; see 633, 634). In herbivorous insects axons show lower membrane potential values near  $-40$  mv (842, 843). Apart from  $K^+$ , other ions may participate in the regulation of these membrane potentials (see 633, 841). Action potentials showing overshoots of 20–30 mv may be recorded in central giant axons of different insects (83, 952; see 633). These action potentials of giant axons depend on a sodium permeability change (83, 688, 689, 843, 915, 952). Sodium ions carry the early inward current, as shown in voltage-clamp studies (687, 688). Tetrodotoxin blocks the giant axon spikes (634, 688, 843).

Related to the problem of axonal  $Na^+$ -dependent action potentials is the problem of the maintenance of an ionic environment rich in  $Na^+$  and poor in  $K^+$  in animals that show either a great variability in their  $K^+$  hemolymph content or a very low  $Na^+$  hemolymph content. Classical experiments by Twarog and Roeder (860) and by Yamasaki and Narahashi (952) show that intact cockroach nerve cord activity may be maintained for hours in saline with low  $Na^+$  content or without  $Na^+$ , but as soon as the nerve cords are desheathed the axon activity deteriorates.

Efforts have been made to resolve this problem, as in phytophagous insect *Carauissius morosus* (see 844), but it is still difficult to decide among the following proposed hypotheses. a) The neural fat body (89, 570), which encapsulates the CNS outside the neural lamellae surrounding the perineurium, would be an important diffusion barrier showing particular  $Na^+$  transport properties. It would concentrate  $Na^+$  inside the CNS (916). b) The perineurium presents tight junctions between its cells and cell processes (573, 776), may act as a barrier, and in some way may behave like the choroid epithelium of vertebrates, regulating the concentration of  $K^+$ , and probably  $Na^+$ , in the CNS by means of a pumping or exchange-diffusion mechanism. This seems the more likely explanation. c) The glial cells would maintain a high- $Na^+$  environment around neurons by some secretion process (776, 841). Desheathing would produce a disruption of their structure (552) and a disappearance of their function and thus deterioration of neuronal activity (776, 844). This possibility seems rather unlikely since it would suppose that insect neuroglia has different properties from neuroglial cells of other invertebrates and vertebrates (see 530, 554).

It is still common to find in textbooks and reviews statements concerning the inexcitability of insect neuronal somata (see for instance 407) in spite of very early successful impalements of cicada motoneurons by Hagiwara and Watanabe (352), who measured resting potentials of  $-60$  mv and orthodromically evoked spikes

showing overshoots of 10 mv. More recently other authors were able to record resting potentials of around -60 mv and spontaneous and evoked action potentials from neurons of the cockroach central ganglia (109, 111, 168, 489-491) and of locust ganglia (168). However, neurons with an inexcitable soma may be observed in insect ganglia (733) and their activity can only be recorded at the neuropile axons (62).

Study of synaptic activity in insect CNS began with the analysis of transmission at the cockroach 6th ganglion by Pumphrey and Rawdon-Smith (704). Extracellular recordings of synaptic potentials were obtained by Yamasaki and Narahashi (951, 953). Recordings of EPSP and IPSP may be achieved by using a gap technique (691) or by impaling either neuronal somas or cell processes at the neuropile (109, 111, 112, 352, 490, 491). A detailed account of general aspects of synaptic activity at the insect CNS may be found in Boistel's review (82).

3) *Acetylcholine*. The possible transmitter role of ACh in the insect CNS has been for years one of the most paper-consuming subjects in the field of invertebrate synaptology. Since detailed accounts of these studies may be found in previous reviews (see 82, 128, 149) only a summary is made here.

A) BIOCHEMICAL DATA. Gautrelet (302) and Corteggiani and Serfaty (153) were first to demonstrate the presence of ACh in insect brains. Since then, variable values of ACh content in insect CNS structures of different species have been reported (see summary in 149).

Even if there are variations in the data obtained by different authors from the same tissue (see 605, 723), the ACh content of all the insect CNS tissues investigated is very high; an estimated content of 500 µg/g tissue was found in *Calliphora* brain (563), and contents between 100 and 200 µg/g wet weight of tissue were found in the brain of *Periplaneta* (770) and *Xyllocopis* (153) and in the nerve cord of *Periplaneta* (143) and *Carausius* (153). The highest content of ACh in insect has been found in a nonnervous element, the royal jelly of *Apis mellifica*, which contains 800 µg/g tissue wet weight.

Many of these values were obtained using bioassay techniques but ACh was also chemically assayed in cockroach nerve cords (see 151). Insect nervous tissue may synthesize ACh in vitro. This has been proven, for example, on cockroach nerve cord (836), blowfly brain (562, 769, 771), and housefly brain (286). The properties and distribution of acetylcholine transferase have been analyzed in insects (see 149); the enzyme may show some structural differences from the enzyme isolated in other phyla (600).

Acetylcholinesterase has been demonstrated in insect CNS by both biochemical and pharmacological methods (see reviews in 128, 149). The Koelle histochemical method shows a rich content and a diffuse distribution of the enzyme (291, 433, 936). The submicroscopic localization of the enzyme using the Barnett technique has been attempted in the nerve cord of *Periplaneta* (778) and in the brain of *Formica lugubris* (550). In the cockroach ganglia a cholinesterase activity sensitive to eserine is present in association with the glial sheaths around both the axons and perikarya and in areas located along the axon membranes within the neuropile. The enzyme deposits are sometimes flanked by clusters of synaptic

vesicles. Glial cells contain a nonspecific cholinesterase (778). In the brain of *Formica* the enzyme appears in some synaptic clefts but also in the cytoplasm of both the pre- and postsynaptic areas (550). These results may be taken very cautiously with respect to the actual localization of the enzyme. However, in the cockroach they may support the idea that the cholinesterase accumulated in the peripheral region of the ganglion may hydrolyze ACh coming from the outside rapidly and thus explain some of the results obtained with ACh perfusion (845; see below).

No precise data about ACh release from insect ganglia by stimulation are available. Early studies attempting a correlation between ACh recovered from the saline bathing ganglia and the global electrical activity recorded from them do not seem conclusive (148, 605; see 149).

b) ACH EFFECTS: THE BARRIER PROBLEM. It has long been known that ACh injected into insects or perfused onto intact nervous systems does not seem to alter either the axon conduction or the synaptic transmission across ganglia (364, 723, 792, 890, 951, 953). A threshold ACh concentration of  $10^{-2}$  M appears necessary to affect transmission across ganglia (951, 953). When the ganglion is desheathed the threshold blocking concentration diminishes to  $10^{-3}$  M (861, 951, 953). When an anticholinesterase drug is added to the ACh solution, blocking effects may be obtained even with  $10^{-4}$  M concentrations (953). More recently, in experiments in desheathed ganglia exposed for long periods to drugs, ACh was reported to act on synaptic transmission from anal nerves to giant axons at much lower concentrations of  $10^{-7}$  M (764). Moreover, either cholinergic antagonists (dTC, hexamethonium) or compounds supposed to block presynaptically cholinergic synapses (botulinum toxin, hemicholinium) blocked transmission across the ganglia in low concentrations (764).

These experiments raise the problem of whether the lack of pharmacological activity of ACh in intact ganglia is due to the presence of a barrier impairing ACh penetration in the ganglia. However, radioactive cations have been shown to enter readily the nerve cord of *Periplaneta* and *Carausius* (see 844) and also [<sup>14</sup>C]-ACh penetrates rapidly into the ganglia of *Periplaneta*. The presence of eserine may slow the process of ACh uptake by the ganglion, perhaps due to a competition from products of ACh hydrolysis (845, 846). Thus the lack of effect of ACh on ganglion elements could be explained by the accumulation of acetylcholinesterase at the periphery of the ganglia. The demonstration that the metabolism of [<sup>3</sup>H]-ACh in the intact nerve cord of *Periplaneta* is very rapid favors this hypothesis (845).

Barrier phenomena exist in insect ganglia for compounds like fatty acids, quaternary ammonium compounds, and alcohol; the penetration of these substances is diminished by large size and polarity, the charged molecules penetrating probably slower than the uncharged molecules of comparable size (227-229, 652). The exact location of these "barriers" has not been elucidated.

c) ACH RECEPTORS AND EXCITATORY SYNAPSES. A better understanding of the action of ACh on neurons of insect CNS has been obtained only recently by using local application of ACh onto the cell membranes either by pressure (111) or by

iontophoresis (111, 490, 491, 695). The effects of the drug on the membrane potential were recorded with an intracellular micropipette.

On quiescent neurons located near the ganglion surface, ACh pulses provoke depolarization and cell firing (111, 490, 491). ACh applications on the soma of spontaneously firing neurons increased the rate of spike discharge (111). Carbachol, nicotine, mecholine, and pilocarpine mimic the effects of ACh (491). When ACh is ejected from a micropipette placed more deeply it may facilitate the discharge of spikes elicited by orthodromic stimulation (111).

The effects of ACh antagonists like dTC on the general activity of an insect ganglion were found to be rather irregular, varying from a total lack of effect even at concentrations  $10^{-2}$  M (723) to a synapse blocking effect in lower concentrations (491, 764, 792).

Insect neuronal responses to local applications of ACh are blocked reversibly by gallamine (695). However, mixed muscarinic and nicotinic properties of binding were found in a brain fraction of *Musca domestica* supposed to contain the ACh receptors (838).

On the other hand, different anticholinesterase agents facilitate the action of ACh on the ganglia (see above) and cause a marked increase of the ganglion global electric activity (149, 491, 605, 723, 724, 861, 951, 953). Escrinc increases the amplitude and duration of the neuronal responses to iontophoretic applications of ACh (695).

A parallel between the ACh responses of cockroach neurons and the EPSP recorded in them has been attempted by Pitman and Kerkut (695). The EPSP's, like the ACh responses, are blocked by gallamine and increase in amplitude and duration under the effects of escrinc. They are also blocked by dTC (111). The reversal potentials of EPSP and ACh responses were found by extrapolation to be around  $-44$  and  $-38$  mv, respectively (695). It seems dubious that these values correspond to the actual reversal potentials.  $\text{Na}^+$  ions seem to be involved in the ACh responses, since these are reduced in amplitude in a low- $\text{Na}^+$  medium.

The pharmacological similarities between the ACh response and the EPSP are interesting, but it seems premature to conclude that there is participation of ACh in the production of the EPSP.

4) *Amino acids.* Glutamic acid and GABA, which have a probable transmitter function at the insect neuromuscular junctions (see sect. ivB), are detected in insect central ganglia. Extraction, chromatography, and ninhydrin treatment of the chromatograms allow the identification of glutamic acid and GABA spots (705, 706). These amino acids are also found in honeybee brain in rather important amounts: glutamate,  $553 \mu\text{g}/100 \text{ g}$  wet weight; GABA,  $109 \mu\text{g}/100 \text{ g}$  wet weight (287, 288).

When *Schistocerca* nerve cords are incubated with [ $\text{U}-^{14}\text{C}$ ]glucose both glutamate and GABA become labeled (91). In nervous tissues from honeybee (288), cockroach (378), and locust (91) labeled glutamate is in part converted to GABA. Glutamic decarboxylase activities have been measured in some of these species and their amount and properties resemble those of the mammalian brain enzyme (91, 288).

If glutamate and GABA are actually involved as transmitters in the neuromuscular junctions, they would be expected to be found in high content respectively in the motoneurons and in the inhibitory neurons to muscle as was observed in *Crustacea* (672; see sect. IV A). Whether these transmitters also play a role in interneuronal connections has not been yet established.

As in the case of ACh, perfusion of GABA to intact ganglia requires very high concentrations ( $10^{-2}$ – $10^{-3}$  M) to be effective (792). In these concentrations GABA appears to block the extracellularly recorded spike activity of the ganglia (768, 890). This block may occur at the level of synapses (768) since axonic action potentials are not affected (297).

Iontophoretic applications of GABA onto neurons located in the periphery of ganglia of *Periplaneta* cause hyperpolarization and inhibition of their spontaneous firing (112, 490, 491, 695). These GABA responses reverse at  $-72$  mv (112, 695). Removing  $\text{Cl}^-$  from the environment or increasing intracellular  $\text{Cl}^-$  causes a reversal of the GABA hyperpolarization (490, 491), showing that GABA action is mainly due to an efflux of  $\text{Cl}^-$ . GABA responses are blocked by  $10^{-4}$  w/v concentrations of picrotoxin (112, 695). The reversal potentials of the GABA response and IPSP recorded from the same neurons are slightly different but IPSP seem to be also blocked by picrotoxin (112, 695). These findings may suggest that GABA is also participating in the synaptic transmission at the CNS neuropile, but further evidence is necessary to verify this hypothesis.

No pharmacological studies on glutamate action on the CNS neurons are available.

5) *Monoamines*. Histochemical studies using the Hillarp-Falck fluorescence technique (see 245) have detected the presence of neurons that possibly contain catecholamines in the brain of *Periplaneta* (289, 290) and *Locusta* (696) and in ganglia of *Schistocerca* (133) and *Annabolia nervosa* (66, 505). The neurons may show yellow-green or green fluorescence and fluorescent varicose fibers are also observed in the neuropile of the brain and ganglia (66, 290). The fluorescence fades in animals treated with reserpine (290). In the thoracic ganglion of *Annabolia*, microspectrofluorometric analysis confirms that the fluorescent neurons actually contain DA (66). Biochemical assay of the ganglion shows that the DA content is 7 times greater than the NE content, but no localization of NE in neurons could be made (66). In *Schistocerca* the amine is claimed to be localized in submicroscopic granulated vesicles 1500 Å in diameter observed in both neurons and axons (133), but no effective proof exists.

The perfusion of catecholamines (DA and NE) to intact or desheathed cockroach ganglia causes either blocking or facilitating effects depending on concentration (389, 861). Application by perfusion of either epinephrine, NE, or DA on cockroach neurons impaled with intracellular micropipettes results in depolarization and excitation of the cells (491). Since DA has been detected in neuropile and neurons, further research on a transmitter function of DA would be worth while.

5-HT may be detected biochemically in cockroach brain and ventral nerve cord (150–152, 308). Homogenates of cockroach brain are able to synthesize 5-HT from 5-HTP (150), but since they also synthesize dopamine from L-dopa (152) it is

difficult to accept the specificity of the decarboxylase. No MAO activity could be detected in cockroach nervous tissue (76, 152). No pharmacological studies on the effect of 5-HT on insect CNS are available.

6) *Endogenous substances and neurosecretion.* Analysis of the possible role of peptides and other substances extracted from the CNS and neurosecretory organs of insects is out of the scope of this review and may be found elsewhere (569, 694).

### B. Neuromuscular Junctions

Synaptic mechanisms in the neuromuscular junctions of insects resemble very much those of crustaceans. Although described separately, it is convenient to keep in mind this unity since the evidence obtained in one phylum complements the data obtained in the other (for previous general discussions see 404, 872, 874). The chemical control mechanism of firefly lantern has been recently reviewed (122).

1) *Structural background.* Although insect muscle fibers may show a large variation in length, diameter, and color, either white or red muscle usually presents the same general structure. They are composed of striated fibers, containing microfilaments of actin and myosin arranged like the muscle fibers of the vertebrates. This resemblance extends to other aspects, such as the structure of both the sarcoplasmic reticulum and the transverse tubular system (for muscle structure see reviews in 35, 702, 774).

Although very different patterns of innervation may be found from one insect to another or even from larva to imago, the submicroscopic picture of different neuromuscular junctions is rather uniform.

The first descriptions of the distribution of nerve endings in insect muscles are due to Doyère (196), Kölliker (508), and Kühne (532). Foetinger (279) later discovered that the innervation of insect muscle fibers was multiterminal, i.e. the branches of the motor axons ended at many points in the same muscle fiber. This finding was confirmed by many workers, the endings being either similar to the end plate of vertebrates, terminating on special elevations of the muscle fibers or "Doyère cones" (35, 359, 400, 619, 746, 834), or being *en passant*, located at many points in the trajectory of the axon (359, 577, 624). Detailed anatomical and histological descriptions may be found in Hoyle (404) and Bullock and Horridge (105).

Indications of a dual axon innervation were found in the beginning of the century by Mangold (574) and the polyneuronal character of insect muscle innervation has been confirmed both anatomically (34, 400) and physiologically (401, 701). The particular problem of inhibitory innervation is discussed below.

Electron-microscopical studies of neuromuscular junctions were performed on different types of muscles in various species: tymbal muscles of *Cicada* (224), body muscles of cockroaches (221), leg muscles of cockroaches (33, 655, 772) and wasps (223), flight muscles of *Cicada* (224), *Tenebrio* (773), *Drosophila* (763), and different species of *Coleoptera* and *Hymenoptera* (361). The structure of nerve-muscle junctions has also been studied in segmental larval muscles in blowflies (664) and waxmoths (56) (see 775, 777).

The general pattern of the junctional ultrastructure appears rather analogous in all these different muscles, the nerves ending either in association with Doyère cones or as a fine branching extending over the muscle surface. The terminal nerve branches, which appear in many cases in a "claw" disposition (400), are "tunicated" (223). They are covered by Schwann cells (lemnoblasts or glial cells) forming concentric or branching mesaxons interrupted by clear spaces (lacunae) in some of their infoldings. The Schwann cell sheath is surrounded by a basal membrane and follows the axon just to level of the ending proper, where it begins to show discontinuities. At these discontinuities the Schwann cell basal membrane coalesces with the basal membrane of the muscle fiber sarcolemma.

Some of these areas are located in protruded regions of the muscle fibers that have been interpreted as corresponding to the Doyère cones of classical histology. In other cases the synaptic regions are constituted by contacts *en passant* along axonal trajects (773). In both kinds of synaptic areas there is a close apposition between the membranes of axon and muscle, leaving a synaptic cleft of around 200 Å. No folds or gutters like those in vertebrate junctions have been seen. The cleft either appears to be filled by an opaque material (763) or presents a desmosome-like appearance (664). In all cases the presynaptic endings contain electron-lucent synaptic vesicles of diameters varying between 200 and 600 Å (according to authors and techniques) and large mitochondria. In some instances large electron-lucent vesicles (773) or "neurosecretory" vesicles (664) have been also encountered. In the postsynaptic region it is sometimes possible to observe dense accumulations of "aposynaptic granules" or complicated whorls of membranes, the "rete synapticum" of Edwards et al. (223). These later features are not observed in every insect junction (773, 777).

2) *Junctional transmission.* When impaled with an intracellular microelectrode the muscle fibers of locust and cockroach show resting potential levels of about -60 mv (63, 344, 352, 397, 398, 867, 878). The membrane potential of locust and cockroach muscle depends mainly on the K<sup>+</sup> permeability of the fiber and the membrane behavior follows the Nernst equation closely when the [K]<sub>o</sub> is changed (344, 397, 398, 871, 942-944). This K<sup>+</sup> electrode behavior of the membrane is observable when K<sup>+</sup> is replaced by Na<sup>+</sup>, maintaining Cl<sup>-</sup> constant. Alteration of [Cl]<sub>o</sub> while maintaining both [K]<sub>o</sub> constant and the medium isosmotic produces only transient changes of E<sub>m</sub> (871, 945). The membrane thus has a finite resting Cl<sup>-</sup> permeability. Moreover, when [K]<sub>o</sub> is increased without maintaining the [K]<sub>o</sub> × [Cl]<sub>o</sub> product constant, an influx of KCl and water is produced (139, 871, 874) and the fiber swells. KCl influx is much faster than the efflux, and after loading the cell with Cl<sup>-</sup> the [Cl]<sub>i</sub> takes a long time to come back to normal (871, 873, 874). These membrane properties are important to remember, since the composition of insect hemolymph in inorganic ions is not homeostatically controlled (see reviews in 278, 840). The implications of this feature for analysis of the synaptic function are discussed below. This variation of the hemolymph content in ions appears even in animals of the same species fed different diets (873, 944).

Direct intracellular stimulations evoke graded local active responses that are propagated decrementally (127). The amplitude and latency of these responses

increase with the strength of the stimulus, showing both refractoriness and undershooting (127). Moreover they are inactivated by sustained depolarization (127, 187), and therefore they are active responses of the electrically excitable membranes. They may be blocked by hyperpolarization, whereas synaptic graded depolarizing responses (EJP's) are increased. Removal of  $\text{Na}^+$  does not abolish the active electrical responses, but diminishes their amplitude (926). Some alkali-earth cations like  $\text{Ba}^{++}$ ,  $\text{Sr}^{++}$ , and less effectively  $\text{Ca}^{++}$  were shown to transform the graded responses of *Romalea* muscles into all-or-none responses showing overshoots. The alkali-earth cations diminish an early increase of  $\text{K}^+$  conductance caused by stimulation, which in normal media would clamp the all-or-none response, and thus graded responses are produced (926). In *Caraussius* divalent cations are believed to carry the current during the depolarizing responses (942). A similar finding has been reported for *Tenebrio* (57) and for some lepidopterans (413).

Some muscle fibers of cockroach and locust muscle may show complete electrical inexcitability (127, 402). These fibers are generally innervated by both slow excitatory and inhibitory axons (879).

Pringle (701) demonstrated that muscles of *Periplaneta* show a double motor innervation. The stimulation of one type of motor axon produces contraction only when it is stimulated repetitively, this contraction being relatively slow. Single-shock stimulation of the other type of axons may evoke contraction of the twitch type. These axons were thus called respectively "slow" and "fast." Stimulation of these axons gives rise to two different synaptic potentials: slow EJP's and fast EJP's, peculiar to each type of axon (54, 127, 187, 397, 400, 401, 878, 879; see 404).

Some muscles are innervated by both types of axon, but sometimes the muscles receive exclusive innervation from only one type (33, 55, 591). This situation has allowed the examination of the ultrastructure of identified nerve endings, but both types of axon terminals have been found to be similar (33).

The EJP's evoked by fast axons in cockroach and locust muscles differ from the slow EJP's. The fast ones consist of large depolarizations (from 1–25 mv, generally between 3 and 10 mv) that frequently reach threshold. The slow EJP's are also depolarizing potentials, though much smaller and seldom firing suprathreshold signals. They may reach threshold by summation (127, 401; see 404). The amplitude of both classes of EJP's is increased by fiber hyperpolarization and decreased when the muscle is depolarized (127, 187). A reversal potential for the EJP's around 0 mv has been calculated by extrapolation from experiments by Cerf et al. (127).

The ionic basis of EJP's has been analyzed in larvae of *Tenebrio* by Kusano and Grundfest (541). The  $E_{\text{EJP}}$  was measured by the current-clamp method or by superimposing the EJP's on directly evoked spikes. In both instances the reversal potential seems to be around 0 mv. This  $E_{\text{EJP}}$  value shifts when  $\text{Na}^+$  is removed, even when  $\text{Li}^+$  is substituted for  $\text{Na}^+$ .  $\text{K}^+$  and  $\text{Cl}^-$  do not appear to contribute to the synaptic current, but increases of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  produce a shift of  $E_{\text{EJP}}$  in the positive direction, showing that they may intervene in the genesis of the EJP's (541).

These results are somewhat different from those obtained in mEJP's recorded

in cockroach muscles, where the reversal potential of the mEJP was calculated by extrapolation to be between  $-10$  and  $-25$  mv (65).

Miniature EJP's may be recorded in resting conditions in muscles of *Schistocerca*, *Blaberus*, and *Periplaneta* (501, 867, 868) *in situ* or *in vitro*. They may be recorded all along the fibers because of multiterminal innervation. They have amplitudes of 200–300  $\mu$ v and the distribution of the intervals between successive mEJP's shows that their frequency curve fits well a Poisson distribution (868). The curve is skewed, as is observed in multiply innervated muscles. Increases in  $[K]_o$  also increase the frequency of the mEJP, decreasing their amplitude.  $[Mg]_o$  above 10 meq/liter decreases both the amplitude and the frequency of mEJP. Increase in  $[Ca]_o$  antagonizes  $Mg^{++}$  effects (868).

5-HT and tryptamine derivatives were found to block both the EJP and the neurally evoked contraction in locust muscles (381). These effects were produced by rather high concentrations of these amines, which do not seem to impair the membrane resistance or the conduction of axonal spikes. The effect of 5-HT has been interpreted as due to a blocking action on transmitter receptors of the postsynaptic membrane (381). Strychnine also blocks neuromuscular transmission in the cockroach muscle in concentrations down to  $10^{-5}$  g/ml, but this blocking action seems to be presynaptic since mEJP's gradually diminish in frequency and disappear (27).

3) *Excitation and glutamate.* The possible participation of ACh in neuromuscular transmission of insects has now been discarded (for review see 149). Application of ACh through different pathways on various species was ineffective in altering junctional transmission (364). Cholinesterase activity does not appear meaningfully related to the neuromuscular junction (360, 433). Moreover, anticholinesterase drugs applied locally do not show any enhancing effects (241, 364, 592, 655, 723, 868).

Many workers have not found effects in neuromuscular junctions of curare applied locally to insect muscles (241, 364, 589, 655, 725). dTC does not affect the mEJP's (868), though recent reports have claimed that dTC injected intra-abdominally may produce both flaccid leg paralysis and a blockade of synaptic activity in leg muscles of different insects (553, 589, 592). Injection of dTC into a leg or the application of the drug directly onto the metathoracic ganglia was also claimed to cause failure of muscle contraction (283). All these effects cannot be due to an action of dTC on the CNS, since dTC is claimed to produce the same effect after the segmental ganglia is removed. The most acceptable explanation would be a direct action of curare on the conduction of impulses in the motor nerves (241, 284).

As in crustaceans (see sect. ivB) there is now an ensemble of evidence supporting the possibility that glutamic acid could be the excitatory neuromuscular transmitter. In locust, grasshopper, and cockroach L-glutamic acid produces membrane depolarization and contraction when applied either locally or systemically (242, 496, 501, 592, 879–881). These contracting and depolarizing effects may be obtained with glutamate concentrations down to  $10^{-8}$  g/ml (242, 592, 880, 881). The optimal concentration producing contraction was found to be  $10^{-5}$  g/ml (880).

Lower concentrations ( $10^{-9}$ – $10^{-12}$ ) are able to potentiate neurally evoked responses and to raise the frequency of mEJP's (496, 880, 881). Glutamate may also cause an increased membrane conductance of locust muscle at concentrations above  $10^{-8}$  g/ml (880, 881). Structure-activity analysis of the effect of short-chain amino acids and related compounds tested on locust, grasshopper, and cockroach muscles shows that L-glutamate is the most powerful excitatory agent of the substances tested. The presence of two acidic groups and one amino group seem to be critical in this respect. Aspartic acid was also active, but in higher concentrations than L-glutamic acid; D-glutamic acid was not active (881).

Iontophoretic applications of glutamate from an external micropipette on locust and cockroach muscles show that only discrete spots on the membrane respond by depolarization to the local application of glutamate (63, 881). These glutamate-sensitive spots seem to coincide spatially with the synaptic areas, as was proven by simultaneous extracellular and intracellular recording (63, 881; see 804). Glutamate receptors show desensitization to repetitive iontophoretic applications of the drug (63, 881). In such experiments, applying repetitive jets, no potentiation was observed (64) as in crayfish muscle (804). Topical applications of glutamate cause desensitization of glutamate receptors, as evidenced by failure in evoking responses in this condition by iontophoretic application. Local application of glutamate may also cause failure of the EJP, this being interpreted as due to crossed desensitization between the natural transmitter and glutamate on the same receptors (881). It is difficult to discard in this case possible effects of glutamate on membrane resistance even if the membrane remains responsive to high-K<sup>+</sup> solutions (881). Glutamate also has presynaptic effects that could explain partially its action on the EJP's (881). However, denervation experiments show that the main action of glutamate is on postsynaptic receptors (63, 881).

The reversal potential of glutamate iontophoretic responses was calculated by extrapolation to be between –10 and –25 mv. This level was similar in the same locust muscles to the reversal potential of the mEJP (63). This result is at variance with those obtained in *Tenebrio* muscles (see above), but the causes of this difference require further investigation.

A) RELEASE OF GLUTAMATE BY STIMULATION. A ninhydrin-positive material found in thin-layer chromatography and identified as glutamate was recovered after neural stimulation of cockroach leg muscles (487). When resting locust retractor unguis nerve-muscle preparations are perfused with saline, a series of amino acids may be recovered from the perfusate. Chemical analysis reveals that the perfusate contains glutamate, aspartate, glycine, and alanine (876, 882). The quantities of amino acids recovered increase when the motor nerves are stimulated, but the increase in the quantity of glutamate released is the most important (876, 882). The amino acids do not seem to come out of the preparation as a result of the neurally evoked muscle contraction. 5-HT, which blocks contraction by acting postsynaptically on the transmission mechanism (381), may double the glutamate release without altering the quantity of the other amino acids recovered from the perfusate. It is difficult to explain these effects of 5-HT on glutamate release (876, 882).

Only the amount of glutamate released seems to be affected by an increase in  $[Ca]_o$ . A two- or threefold increase in  $[Ca]_o$  enhances the release of glutamate, but no changes in the glycine and aspartate content of the perfusate appear (876, 882). An increase in  $[Mg]_o$  depresses the release of all amino acids to such a level that they cannot be assayed in the perfusate; this disturbing fact remains unexplained.

Only glutamate release seems to be dependent on the frequency of nerve stimulation (876, 882). The glutamate release increases proportionally to the frequency, attaining an optimum when the nerves are stimulated at 15/sec. Beyond this frequency the release decreases (882).

Despite the lack of electrophysiological controls in these experiments (see 520), they may be accepted as an indication that glutamate is released by nerve stimulation in locust neuromuscular junctions.

b) INACTIVATION OF GLUTAMATE. There are various enzymes that could metabolize glutamate: glutamic decarboxylase, glutamic transaminase, and glutamic dehydrogenase. The effects of some of these enzymes and their inhibitors in high concentrations ( $10^{-3}$  g/ml) on neurally evoked contractions were studied on the retractor unguis of the locust (881). The results obtained are so complex and confusing that they do not permit any serious conclusion about the participation of these enzymes in the inactivation of glutamic acid and the natural transmitter.

Glutamate decarboxylase is present in the CNS of some insects (see sect. vA). The enzyme is present and plays an important role in GABA synthesis in crustacean peripheral inhibitory nerves (see sect. ivA, B) but there are no available data on its presence in insect nerves. The physiological data about a possible inhibitory transmitter role of GABA in insect muscle inhibition (see below) suggest that this is likely. Nevertheless, since only a part of the muscle fibers actually receives inhibitory inputs (401, 879), it is difficult to believe that glutamate would be inhibited enzymatically at the synaptic cleft.

Since glutamate uptake in crustacean neuromuscular preparations has been demonstrated (431), it is likely that if glutamate is actually a transmitter it may be taken up by tissue elements of the junctions. Recently Faeder and Salpeter (243) found by chemical and autoradiographic methods that neuromuscular preparations of the cockroach metathoracic adductor muscle have a high affinity for [ $^3H$ ]-glutamate and that less than 1% of the labeled amino acid is incorporated into proteins. Nerve stimulation increases the uptake (243). The average tissue concentration bound during a 1-hr incubation period in  $10^{-5}$  M [ $^3H$ ]-glutamate after nerve stimulation was  $2.8 \times 10^{-5}$  M (243). In electron-microscope radioautographs the Schwann cells appear to contain the highest concentration of all the tissue elements in the preparation (for instance the sheath-axon ratio was 2.8). The label appeared on both the surface and the interior of the muscle fibers, although it seemed more concentrated over the nerve ending than over the axon trajectory (243). The junctional region was the most labeled spot of all the tissue (243).

These results indicate that glutamate is taken up by insect neuromuscular preparations. What seems to be less sure is the localization of the process, because of the possible influence of glutaraldehyde fixation in the solubility and localization

of the amino acid. However, these results considered together with the findings of glutamate uptake in crustacean junctions (431, 662) indicate that an uptake mechanism could explain the rapid inactivation of glutamate in the case of a release into the synaptic cleft. It recently has been suggested that this uptake mechanism may also contribute to maintenance of a low level of glutamate in the junctional area (243).

c) PROBLEM OF GLUTAMATE UBIQUITY. The blood of many insects contains high concentrations of amino acids (793; see 278, 840). Usherwood and Machili (880, 881) found in the hemolymph of the locust a glutamate concentration ranging between  $3.7 \times 10^{-4}$  and  $5.4 \times 10^{-4}$  M. Such a concentration has been shown (see above) not only to cause depolarization and contraction of the muscle fiber, but also to cause a serious impairment of the membrane resistance and to desensitize the glutamate receptor. These facts would be incompatible with a transmitter function of glutamate in insect muscle. The same argument has been raised for crustaceans (see sect. iv).

Usherwood and Machili (881) rejected this objection in simple and elegant experiments. When saline containing 10 mM K<sup>+</sup> bathing a locust nerve-muscle reparation is replaced by hemolymph, no change in neurally evoked contractions is observed. If instead of hemolymph normal Ringer is added with the same quantity of glutamate normally contained in the hemolymph (i.e. 10 g/liter), the neurally evoked contraction fades and disappears (881). Since in both cases the same glutamate content is present in the bath, it must be concluded that the glutamate content of the hemolymph is not in a free form, and the objection that the ubiquity of glutamate would impair its transmitter function appears invalidated (876, 881).

d) GLUTAMATE SUPERSENSITIVITY IN DENERVATED MUSCLES. After nerve section the distal stump of peripheral insect nerves undergoes rather rapid degeneration (64, 80, 725). Insect muscle fibers may show degeneration after denervation (863), accompanied by early failure of neuromuscular transmission (869, 877), and decay and suppression of miniature activity if no reinnervation appears (870). The synaptic endings rapidly loose their vesicles and the mitochondria swell and clump (877).

It is well known that after denervation vertebrate muscle becomes more sensitive to ACh. ACh receptors normally located on the end plate, and on a restricted region around it, extend to the whole muscle surface after denervation (40, 606).

Denervation of the retractor unguis muscle of the locust also induces extension of glutamate receptors to areas normally insensitive to the amino acids (875, 876). This interesting finding emphasizes the likelihood of an excitatory transmitter function of glutamate in insect muscle.

In summary, glutamate looks like a good candidate for the transmitter of excitation from nerve to muscle in insects: it is released from terminals by nerve stimulation, it mimics well the effect of the transmitter on the ionic conductance of the membrane, and it could be rapidly inactivated by uptake. Moreover the denervated muscle shows hypersensitivity to glutamate, as would be expected from a transmitter.

Further data are necessary to show that the insect motoneurons are chemically similar to those of crustaceans. It would also be interesting to have some pharmacological

logical means of demonstrating the identity of glutamate receptors as those of the natural transmitter.

4) *Inhibition and GABA.* The inhibitory innervation to insect muscles was first suggested by Friedrich (285) and supported by later reports (54, 714). However, this evidence was considered dubious for years. Hoyle (401) studied the inhibitory innervation of locust muscles and first recorded hyperpolarizing junctional potentials evoked by nerve stimulation, but without accepting their inhibitory character since no attenuation of the EJP's by the hyperpolarizing potentials could be detected. Hyperpolarizing IJP's have been recorded since then in muscle fibers of locust, grasshopper, cockroach, and beetle and were finally accepted by many workers as true IJP's (65, 419, 420, 422, 423, 873, 878, 879).

In different species of insects [e.g. *Romalea* and *Locusta* (127, 401, 879)] the inhibitory axons only innervate the muscle fibers that are also innervated by the excitatory slow axon. Many of these fibers may be electrically inexcitable (879). The IJP's in these fibers are generally hyperpolarizing but in special cases they may appear to be depolarizing (405, 406). They generally have amplitudes of 1–25 mv, being larger in the muscles fibers that show a low resting potential. The IJP's may also vary in duration between 40 and 300 msec (879). The changes in conductance causing the IJP seem to persist as long as the hyperpolarization (879).

In preparations of locust muscle *in situ*, the value of the  $E_{IJP}$  may vary according to fibers, fluctuating between -55 and -75 mv, but an average of -70 mv is generally measured. This  $E_{IJP}$  value was shown by Usherwood and Grundfest (879) to probably correspond to  $E_{Cl^-}$ . When all the  $Cl^-$  content of the medium bathing locust muscle is replaced by an impermeant anion, such as propionate, the IJP's reverse their sign. However, the quantitative aspects of these experiments were difficult to analyze in such an *in situ* preparation. They could be better examined in an isolated preparation of the anterior coxal adductor of the cockroach. In such a preparation the IJP's are generated only by  $Cl^-$  ions and changes in  $[K]_o$  do not directly affect the  $E_{IJP}$ . As described above, changes in  $[K]_o$  may alter  $[Cl]_i$  because of water movements and thus indirectly influence  $E_{IJP}$  (873, 879).

GABA has the same action on locust and cockroach muscle as on crustacean muscle, producing a fall in membrane resistance and hyperpolarization in concentrations down to  $10^{-8}$  g/ml (490, 879). GABA also produces a diminution of the EJP's. Picrotoxin blocks the effects of GABA on the membrane potential, the membrane resistance, and the EJP's, but without affecting them itself (879). Moreover, picrotoxin blocks the IJP's at concentrations of  $10^{-5}$  g/ml. GABA responses reverse at the same membrane level as the IJP's.  $E_{GABA}$  is equal to  $E_{Cl^-}$  and is not affected by variations in  $[K]_o$ , except when these variations affect  $[Cl]_i$  (879). High concentrations of picrotoxin ( $10^{-3}$  g/ml) may have a presynaptic effect and may affect neurally evoked contractions (879).

The effects of the inhibitory stimulation on the EJP's evoked by stimulation of the fast motor axons are minimal, since both axons never innervate the same fibers, and naturally the same happens with neurally evoked fast contractions. The stimulation of inhibitory axons only depresses the contractions elicited by the stimulation of the slow axon (873, 879). As expected, GABA has no effect on the

contraction elicited by the excitation of the fast axon, and the fibers not innervated by inhibitory axons apparently are not endowed with GABA receptors (879).

The actual character of stimulation of inhibitory axons in the anterior coxal adductor muscle of locust and grasshopper was challenged by Hoyle (405, 406), who found that inhibitory axons were not always actually inhibitory, since sometimes their stimulation may cause depolarization and excitation of the muscle. Hoyle (405, 406) preferred to consider these nerves as "inhibitory-conditioning" nerves rather than true inhibitory nerves. Usherwood (873) proved that the recording of depolarizing IJP is either due to the entry of KCl inside the muscle during dissection of the preparations or to the high (10 mM) K content of the hemolymph of the grassfed locusts. In starved locusts the IJP's are generally hyperpolarizing. These variations in KCl content may induce changes in  $[Cl]_i$  that could account for the diversity of responses recorded by Hoyle (406).

Specific information regarding the distribution of GABA receptors is still lacking. Dose-response curves relating GABA action with its effects on membrane conductance indicate that to produce the change in conductance three molecules of GABA must interact with a single receptor site (925). These results are different from the observations on the crustacean neuromuscular junction (see 252).

Unfortunately, there are available neither biochemical data on insect inhibitory motor neurons nor information about the release and uptake of GABA by insects.

If some extrapolations from the data obtained in inhibitory junctions of crustaceans are allowed, GABA is a likely candidate for inhibitory transmitter in insect neuromuscular junctions. Perhaps in the next few years the present information gaps will be filled.

## VI. MOLLUSCS

### A. Central Nervous System

Among invertebrates the molluscan CNS ganglia are probably the best known regarding the chemical transmitters involved in its operation. Unfortunately this knowledge is not paralleled by available information about their organization or cell architecture. Although maps of some molluscan ganglia are available that chart a limited number of identifiable neurons, we ignore almost completely the precise function of the nerve cells and the connections existing among them and with the periphery.

The reader will probably find in this section an exaggerated emphasis on findings obtained in a small number of gastropod species. This bias results from the evolution of the research in this field and not from the "professional deformations" of this reviewer.

1) *Structural background.* A detailed account of the general anatomy and morphogenesis may be found in Bullock and Horridge (105). The following short description refers mainly to the CNS of gastropod and bivalve molluscs. Cephalo-

pod synapses are mentioned for comparison. The giant synapses of the squid are treated separately (sect. viB).

Gastropod central ganglia generally show in their periphery a multilayered arrangement of neuronal bodies that send their axons to the core of the ganglia, where they branch and form a very complex neuropile. Gastropod central neurons are generally unipolar (see 19, 102, 175, 535, 647), but some bipolar neurons have been described (747). The somata are rather voluminous, with average diameters of 100–200 Å. The giant neurons of *Aplysia* may reach diameters of almost 1 mm. Glial cells surround the neurons and their prolongations form a complex tropho-spongium around the somata. Glial infoldings penetrate deeply in corresponding invaginations of the surface of the perikarya and axons (see 46, 141, 639). Glial cells are also observable at the neuropile, the nerve connectives, and the peripheral nerves, and they may closely follow even the thinnest axons (8, 141, 253, 637, 640, 729, 739, 748; see 638). The majority of the central synapses of *Helix* and *Aplysia* are axo-axonal and the somata are devoid of synaptic contacts (102, 141, 310, 729). Axosomatic synapses have been observed in the cerebral ganglia of *Anodonta* and *Elliptio* (437, 965, 968) and in the procerebrum of *Helix* (971, 972). The classical synaptic morphology of vertebrate synapses showing membrane differentiation on both sides of the synaptic cleft is rather rarely found in *Aplysia* and *Helix* visceral ganglia (141, 309, 310, 729). In this case, synaptic contacts perhaps may be localized at the regions where synaptic vesicles attach to the inner surface of the presynaptic membranes. Synaptic structures showing the differentiations of vertebrate synapses have been described as of frequent observation in *Archachatina* (8), *Anodonta* (965), and in *Octopus* brain (see 339, 340, 444, 445).

The synaptic endings observed in the neuropile of the different molluscs examined show, apart from mitochondria and particulate glycogen, a very complex population of synaptic vesicles. Three main types of vesicles may be recognized (309, 310, 729): *a*) small "clear" vesicles 500–800 Å in diameter resembling synaptic vesicles found in the vertebrate neuromuscular junctions and other locations; *b*) granulated vesicles showing a dense core (these are slightly larger than the clear vesicles and may be compared to catecholamine-containing vesicles of adrenergic endings); and *c*) large vesicles that may reach diameters of 1400 Å, showing either an electron-opaque core or a multivesicular content. They have been homologized to elementary neurosecretory granules found in neurosecretory systems.

However, as Coggesshall (141) has pointed out, other types of granules and vesicles may be observed that range between 500 and 2500 Å in diameter and are difficult to fit in the three categories described. Moreover it is also possible to visualize inside the terminals coated vesicles formed by pinocytosis (129). Many synapses contain clear vesicles alone (8, 141, 310, 442, 443, 729, 971) and it is tempting to ascribe a cholinergic function to these endings (310). Indirect support of this assumption could be the richness in ACh of synaptosomes containing clear vesicles isolated from *Octopus* brain (443).

The synapses that show a majority of granulated dense-core vesicles may also contain clear vesicles (141, 310, 967). There is no indication whether they correspond to different types of vesicles or to evolving forms of the dense-core type of

vesicles. No firm evidence exists about the contents of dense-core granulated vesicles. Perhaps they contain dopamine (see below), but the pharmacological experiments attempting to induce changes in their number or their content (967) are inconclusive. The granulated dense vesicles may also be found in neuronal somata and along axons (141, 968).

The endings containing the vesicles that resemble elementary neurosecretory granules may also contain clear vesicles (310). As Simpson et al. (765) pointed out in their survey on neurosecretion in gastropods, the presence of elementary neurosecretory granules does not imply a neurosecretory function for the structure. These so-called elementary neurosecretory granules may also be observed in molluscan neuronal somata and along axons (8, 141, 551, 766, 923).

In *Aplysia californica* two kinds of neurosecretory cells have been described (141, 282). The first type, the so-called "white" neurons, probably correspond to the same neurons that Scharrer (756, 757) previously described as neurosecretory in *Aplysia*. These cells may be stained using techniques that stain neurosecretory neurons in other tissues; they are almost unaffected by neural inputs; and they possibly send their axons to the ganglion envelopes, which have been considered as a possible neurohemal area (141, 146, 282). Similar neurohemal areas located in the sheaths of ganglion connectives or of different peripheral nerves have been described in other gastropods (649, 766, 923).

The white cells may be activated by chemical stimulation of the osphradium using extracts of seaweed, oyster, and shrimp pellet (435). Osmotic or mechanical stimulation of the osphradium affects them only transiently (435). The secretory function of the white cells remains unknown.

The second group of neurosecretory cells in *Aplysia* are the so-called "bag cells." The evidence for considering these cells as neurosecretory seems more solid. The neurons are localized in clusters at points where the left and right pleurovisceral connectives enter the visceral ganglion. They are isolated from the proper visceral neurons by encapsulating connective tissue (141, 282, 538). Each cluster contains about 400 cells in adult animals. The cells contain large vesicles 1500–2000 Å in diameter, each with an electron-opaque granule inside. The bag cells send processes to the sheath enveloping the nerve connectives and the ganglia (141, 789). All the bag cells show the same electrophysiological properties. They are normally silent and unresponsive to stimulation of peripheral nerves, but the stimulation of the connectives provokes an apparently synchronous activation of all the cells of the cluster consisting of a prolonged repetitive spike discharge that may last for long periods (538). After cessation this discharge may only be reproduced after a period of 1 hr or more (538).

Homogenates of bag-cell clusters injected into *Aplysia* cause egg-laying activity after a latency period of 1 hr (536, 537, 789). Extracts of the tissue remaining from the ganglion or the connectives after removal of the bag-cell clusters may result in some egg-laying activity (537, 789), but this is much less important than the one caused by the bag-cell extract (537). This residual activity may be attributed to axons and endings of the bag cells remaining in the ganglion sheaths (789). After electrical stimulation of the connectives, the perfuse of the abdominal ganglion

is able to induce egg-laying. This has been interpreted as due to a secretion release from the bag cells (537) but other substances may also be released by nerve stimulation. A more interesting finding is the isolation of a specific protein uniquely located in bag cells that induces egg-laying when injected into *Aplysia* (837). The bag-cell extracts or the bag-cell specific protein would act by causing contraction of small muscle cells that surround the ovotestes, thus causing the expulsion of ovocytes (142).

2) *Identification of neurons.* In some species of gastropods the identification of neurons by their localization and electrophysiological properties has been attempted. However, such maps are only a first important step toward the determination of the actual functions of the neuron. These studies have been particularly successful and useful in *Aplysia* (15, 18, 19, 141, 282, 299, 415, 416, 472) and *Tritonia* (939).

Arvanitaki et al. (15, 18, 19) were able to consistently identify 7 neurons of *Aplysia* visceral ganglia, and more recently Frazier et al. (282) completed a map of the larger neurons (around 30 perikarya) and of 8 cell clusters, which may be visualized on both the dorsal and the ventral faces of the ganglion. Some other 13 cells may be identified, but not as individual entities. The identification is based on morphological criteria (position, size, pigmentation) and electrophysiological criteria (firing, inputs, connections, spread of axonic branches, and responses to ACh). Each surface was divided in four quadrants and the cells named with a letter according to right (R) or left (L) position and arbitrary numbers. The same principle was used for the classification and identification of neurons in the bilateral buccal ganglia of *Aplysia* (299), where 10 symmetrical neurons may be identified in each side.

In the pleural ganglion of *Aplysia* Kehoe (472) described three main clusters of neurons: the anterior pleural neurons (APL), the medial pleural neurons (MPL), and the posterior pleural neurons (PPL). The neurons composing each of these given clusters do not vary much in size or shape and show similar electrophysiological properties, including a similar synaptic input (see below).

Identification and mapping of neurons have been also attempted in *Helix aspersa* (334, 484) and *Lymnaea stagnalis* (742) but the usefulness of such maps has not been yet demonstrated.

3) *Properties of neurons.* Molluscan neurons may be impaled easily by one or more microelectrodes in vivo or in vitro. When so impaled they show resting potentials varying between -40 and -60 mv according to species and neuron size. The membrane potential may only be predicted from the Hodgkin-Katz-Goldman equation when the temperature is lower than ~5 C (336). In normal conditions at room temperature (20-22 C) an electrogenic Na<sup>+</sup>/K<sup>+</sup> pump probably contributes to some extent to the regulation of the neuronal membrane potential in *Helix* (499, 622, 832), *Anisodoris* (336), and *Aplysia* (123). Intracellular injection of Na<sup>+</sup> (see 832) or even spike discharge (see 621) may activate the pump, causing some hyperpolarization.

Molluscan neurons may discharge action potentials spontaneously. An analysis of the different types of endogenous spike activities is outside the scope of this review

and can be found elsewhere (17, 788, 816). Action potentials of the giant neurons of *Aplysia* evoked orthodromically, antidromically, or by intracellular stimulation generally take origin in a region located beyond the neuronal axon hillock and then secondarily invade the somata (820). However, in pacemaker neurons the somata may also generate action potentials when isolated from the axon (7). The action potential of molluscan neurons may be recorded in a Na<sup>+</sup>-free medium in the presence of Ca<sup>++</sup> (125, 304, 305, 307, 425, 603, 656). Voltage-clamp studies show that the early inward current may be observed in Na<sup>+</sup>-free solutions in the presence of Ca<sup>++</sup> (131, 303, 521, 522, 572).

Synaptic activity and integration in molluscan ganglia have been extensively analyzed and will only be considered here in relation to chemical transmitters. For data in synaptic organization and plasticity the reader may consult previous reviews by Tauc (821-823), Kandel and Spencer (453), and Kandel and Kupfermann (452).

4) *Acetylcholine*. There is now a certain amount of evidence regarding the transmitter role of ACh and its implications in the organization of the molluscan CNS.

A) BIOCHEMICAL DATA. In early screenings for ACh in invertebrates it was found that *Aplysia* central ganglia contained ACh and that the cerebral ganglia of *Octopus* were the nervous structures richest in ACh (41, 43; see 42). This was confirmed later in the ganglia of many species of gastropods and cephalopods (251, 265, 483, 503, 564, 914). The localization of ACh in the cytoplasm of individual neurons in *Aplysia* follows from the data on the distribution of the synthesizing enzyme (see below). The demonstration of its localization at the level of molluscan synaptic endings could only be obtained in *Octopus* brain (276, 443) by isolating synaptosomal fractions showing ACh contents 100 times greater than the corresponding fractions of mammalian brain. It is also known from early studies on the enzyme that some molluscan nervous tissue (ganglia of *Loligo* and *Sepia*) contains choline acetyltransferase, the enzyme synthesizing ACh from choline and acetyl-CoA (251, 630). This has also been confirmed in different ganglia from *Aplysia* and *Helix* by studies at the cellular level (166, 330, 331, 587). Giller and Schwartz (330, 331) dissected out of the visceral ganglion of *Aplysia californica* each of the 30 neurons identified by Frazier et al. (282) and assayed them individually for the enzyme after incubation of the ganglion with [<sup>14</sup>C]-labeled choline and acetyl-CoA. Only 3 of them (L10, L11, and R2) were found to contain the enzyme. The activity of the 3 neurons together corresponds to  $\frac{1}{3}$  the total activity of the visceral ganglion. McCaman and Dewhurst (587) confirm this finding, but obtain lower activity values for each cell. Homologous cells, such as the pleural and the visceral giant cells, show the same enzyme activity (331, 587).

Cholinesterase with the characteristics of specific acetylcholinesterase has been detected biochemically or histochemically in the nervous ganglia of different molluscs (42, 95, 175, 193, 235, 509, 636, 638). The problem that does not seem solved is localization of the enzyme. Identified cholinergic neurons of *Aplysia* seem to contain the same amount of acetylcholinesterase as identified noncholinergic neurons (332, 588). On the other hand, the neuropile of the ganglia appears to show more cholinesterase activity than the neurons (332). This is perhaps an indi-

cation that the enzyme is preferentially localized at the synaptic regions. Such a localization also has been postulated on the basis of electron-microscope histochemical studies (636) but the resolution of the methods used allows no precise localization of the enzyme.

B) ACh RECEPTORS. Most information on cholinergic synapses in molluscan CNS comes from studies on sea slugs and land snails. These studies were initiated by Tauc and Gerschenfeld (827-830; see 313, 822), stemming from the observation that when ACh is perfused in low concentrations on *Helix* and *Aplysia* ganglia it may cause opposite effects; it depolarizes and excites some neurons (D-response) while hyperpolarizing and inhibiting other neighboring ones (H-responses) (828, 829). In this review reference is made to D- and H-responses (or receptors) instead of D- and H-neurons, as originally used. The original pharmacological classification of the neurons, which involved a series of acronyms (325; see 312), has been useful in the development of research but probably has become obsolete due to the complex patterns and combination of receptors more recently demonstrated even for single neurons (see below).

Iontophoretic applications of ACh onto the somatic membrane of molluscan neurons generally reproduce the responses obtained by perfusion (451, 469, 472, 828, 829). Of all the ACh effects described in different gastropods three main responses appear to be most common: a) H-responses that reverse at about -60 mv, b) a second type of H-response that reverses at about -80 mv, and c) D-responses (469, 472, 828-830). The time courses of the H-responses obtained for the same dose of ACh are not the same (469, 472); the H-response that reverses at about -60 mv is shorter and is described as rapid H-response. The H-response that reverses at about -80 mv lasts much longer and is called the slow H-response. The cause of these differences in time could be a different time of binding of ACh to the receptors involved in each H-response. It is difficult to think that AChE could be involved in the difference since both responses may be evoked by applications of ACh onto the soma.

Since the early studies, it was found easy to discriminate pharmacologically between the ACh receptors involved in the D-response and in the rapid H-response, at least in *Aplysia* and *Helix*. d-Tubocurarine and atropine block both kinds of receptors (829, 830), but hexamethonium selectively blocks the receptor involved in the D-responses without affecting the receptors of the rapid H-responses (472, 829, 830). On the other hand, the ACh receptors involved in the slow H-responses observed in *Aplysia* neurons are completely different from the other two types: they are not affected by dTC, atropine, or hexamethonium (467, 470, 472) but may be selectively blocked by tetraethylammonium or methylxylocholine (470, 472).

The D-responses and the rapid H-responses have been observed in many gastropods: *Aplysia* (325, 451, 472, 751, 828), land snails like *Helix* or *Cryptomphallus* (135, 136, 497, 827), *Onchidium verruculatum* (346, 657, 752). Slow H-responses have been only reported in *Aplysia* ganglia so far (469, 472). In *Navanax* an H-response reversing near -55 mv recently has been observed (559) but it is not affected by the agents that block the rapid H-response in other gastropods.

ACh receptors of molluscan neurons have an ACh sensitivity resembling the

ACh receptor of neuromuscular end plates (see 188). They may respond to concentrations of  $10^{-14}$  M ACh, giving peak responses of some millivolts (322, 323).

ACh receptors show desensitization (826). When ACh is perfused or repetitively applied by pulse iontophoresis, the responses decay in amplitude with the prolongation of perfusion or with repeated ACh pulses. These desensitization phenomena resemble those originally described by Katz and Thesleff (468) in the vertebrate end plate.

c) IONIC BASIS OF ACH RESPONSES. The difference between the reversal potentials of the rapid and the slow H-responses is a good indication that the ionic mechanisms involved in their generation are different.

There is now rather good evidences from *Helix* (498), *Cryptomphallus* (135), *Onchidium* (657, 752), *Aplysia* (74, 75, 469, 473, 751), and *Navanax* (559, 560) that the rapid H-responses are due to an increase of the neuron membrane permeability to  $\text{Cl}^-$ . In snail neurons the complete replacement of  $\text{Cl}^-$  by an impermeant ion like  $\text{SO}_4^{2-}$  causes the reversal of an H-response due to a shift of  $E_{\text{ACh}}$  (135, 498). In *Aplysia* neurons the replacement of half the  $\text{Cl}^-$  content of the normal seawater bathing the cells by sulfate or methylsulfate causes a 17-mv shift of  $E_{\text{ACh}}$  as predicted by the Nernst equation (469, 472). The intracellular injection of  $\text{Cl}^-$  ions also produces a reversal of the H-response (280, 319, 469, 473, 498). Change of the  $\text{K}^+$  content of the medium has no effect on the reversal potential of H-responses in *Cryptomphallus* or *Aplysia* (135, 473). That  $E_{\text{ACh}}$  corresponds to  $E_{\text{Cl}}$  in neurons showing Cl-dependent H-responses may be also tested by calculating the values of  $E_{\text{Cl}}$  in different molluscan neurons from measurements of the internal chloride activity ( $a_{\text{Cl}}$ );  $a_{\text{Cl}}$  was measured with chloride silver electrodes in *Helix* (488), *Aplysia* (38), and *Onchidium* (658). Microelectrodes filled with an ionic exchanger rather selective to  $\text{Cl}^-$  were used by Brown and his colleagues to measure  $a_{\text{Cl}}$  in *Aplysia* (533, 534, 737, 896). In snail neurons  $[\text{Cl}]_i$  was found to be about 8 meq/liter (488), a value that fits rather well the theoretical value of 12 meq/liter calculated from the Nernst equation by assuming that  $E_{\text{ACh}} = E_{\text{Cl}}$  in neurons showing rapid H-responses (135).

In *Aplysia* the  $[\text{Cl}]_i$  value calculated by Kehoe (473) from the  $E_{\text{ACh}}$  of -60 mv is about 53 mM, which is not very different from the value of 43 mM measured with silver electrodes (38). Moreover, the  $E_{\text{ACh}}$  value of -60 mv measured for *Aplysia*  $\text{Cl}^-$ -dependent H-responses (469, 473, 830) does not differ from the calculated  $E_{\text{Cl}}$  values of -57 mv using the intracellular Cl activity values measured with an ionic-exchanger-filled micropipette (533, 534) in some identified neurons of *Aplysia* (L1-L6) showing rapid H-responses. In analogy to the ionic channels of vertebrate motoneurons (see 214, 333) the ionic channels activated in the  $\text{Cl}^-$ -sensitive H-response in *Helix* were found permeable only to a series of intracellularly injected anions whose hydrated radii are less than 3.15 Å, with a still unexplained exception of the formate ion (498). In *Aplysia* neurons, propionate and isethionate do not behave as completely impermeant anions (472).

From these results it may be concluded that the ACh rapid H-response is exclusively  $\text{Cl}^-$  dependent and that  $E_{\text{ACh}} = E_{\text{Cl}}$ . However, the values of  $E_{\text{Cl}}$  in *Aplysia* neurons calculated from the measured values of  $[\text{Cl}]_i$  show still unexplained variations of 5-10 mv (533, 534, 896) but generally they are more negative than the

resting membrane potentials of the same neurons. This may be an indication that  $\text{Cl}^-$  is not distributed passively across the membrane and that some other mechanism may intervene in its distribution, such as a metabolic pump (135, 498, 657). This seems to be the case for the squid giant-axon membrane (504).

The ionic mechanism of the slow H-response is different and depends exclusively on a selective change of the membrane permeability to  $\text{K}^+$  (469, 472). Neither the intracellular injection of  $\text{Cl}^-$  nor the alteration of the  $\text{Cl}^-$  content of the environment affects the amplitude or the reversal potential ( $E_{\text{ACh}} = -80 \text{ mv}$ ) of this H-response (473). Changes in  $[\text{K}]_o$  cause shifts of the  $E_{\text{ACh}}$  as predicted for  $E_K$  by the Nernst equation (473). Cesium ions do not replace  $\text{K}^+$  in generating the slow H-response, but the membrane appears to be half as permeable to  $\text{Rb}^+$  as it is to  $\text{K}^+$  (473). TEA and cesium ions injected intracellularly block the slow H-response, probably by blocking the  $\text{K}^+$  conductance (469, 470, 473). TEA applied extracellularly also blocks the response, but probably by acting mainly as a cholinergic blocking agent (470, 473), since other  $\text{K}^+$ -dependent drug actions such as a dopamine hyperpolarization of the same cells are not affected by external TEA (21) (see below).

The studies on the ionic mechanism involved in the D-responses have been confused by the fact that in *Aplysia*, *Onchidium*, and land snails two different reversal potentials have been found for the depolarizing actions of ACh: one observed around  $-25 \text{ mv}$  (136, 280, 653, 657) and another that could not actually be observed but was calculated to be near the zero potential level (75, 136, 751). There are now some good arguments that the depolarizing responses reversing around  $-25 \text{ mv}$  result from the activation of more than one ACh receptor and this is described further below. Therefore, the actual D-responses are those whose reversal potentials cannot be directly observed because the delayed rectification phenomena will not permit enough current to pass to reach the actual  $E_{\text{ACh}}$ . Extrapolation to a null value of straight lines obtained from plots of D-response amplitude against  $E_m$  yields an  $E_{\text{ACh}}$  value of about  $0 \text{ mv}$  (136, 751). These D-responses mainly depend on a change in the membrane permeability to  $\text{Na}^+$ ; replacement of part of  $[\text{Na}]_o$  by sucrose or tris-Cl diminishes the amplitude of the responses and shifts the value of  $E_{\text{ACh}}$  (75, 136, 472, 751). Alteration of the  $\text{Cl}^-$  content of the environment does not affect the response amplitude or the  $E_{\text{ACh}}$  (135, 751). A possible participation of  $\text{K}^+$  cannot be completely discarded and data regarding effects of replacement of  $\text{Na}^+$  by other cations are still lacking.

The depolarizing responses that reverse around  $-25 \text{ mv}$  seem to depend mainly on  $\text{Cl}^-$  (136, 280, 752). Replacement of  $\text{Cl}^-$  by sulfate increases the responses by shifting the  $E_{\text{ACh}}$  to a more depolarized value near  $0 \text{ mv}$ . Further removal of 75% of the external sodium by replacing  $\text{NaCl}$  by sucrose does not differ markedly from the effects of  $\text{Cl}^-$  removal alone (136).

This situation would imply that the same chemical transmitter changing the same ionic permeability on two different neuronal membranes would produce opposite effects (136, 488). The main difference between these neurons, as may be easily shown by the Nernst equation, would reside in their internal  $\text{Cl}^-$  concentrations.

It already has been mentioned that some variation in the  $[\text{Cl}]_i$  of molluscan

neurons has been reported (38, 488, 896). However, none of the values of  $E_{Cl}$  calculated from these  $[Cl]$ , measurements explains the equality  $E_{ACh} = E_{Cl} = -25$  mv. Kehoe (472, 473) recently reported that in *Aplysia californica* neurons that show apparently single responses to ACh have reversal potentials values intermediate between the ACh reversal potentials levels for the three ACh responses. Further pharmacological study of these responses demonstrated that they actually correspond to a complex ACh action on multiple receptors, thus activating different ionic channels (472, 473). Following this line, preliminary experiments on depolarizing responses to ACh reversing around -25 mv in *Aplysia* and *Helix* neurons show that after perfusion with hexamethonium, which blocks the D-responses (see above), a depolarizing response reversing at -25 mv is converted to a  $Cl^-$ -dependent H-response reversing around -55 mv to -60 mv (22). This finding clearly indicates that the depolarizing responses reversing at -25 mv are actually superimposed multireceptor responses; therefore the only authentic D-responses are the  $Na^+$ -dependent ones.

D) PHARMACOLOGY OF ACH RECEPTORS. ACh receptors on gastropods generally require, to be activated or inactivated, higher concentrations of agonists or antagonists than vertebrate preparations (see Table 1). Since the early work in these receptors it seemed difficult to fit them into the classification of nicotinic or muscarinic receptors of vertebrates, because it was found that both atropine and dTC could block the same receptors equally (829, 830). Kehoe (472) recently analyzed this problem thoroughly in the pleural ganglion of *Aplysia*, concluding that the receptors mediating both the  $Cl^-$ -dependent inhibition and the  $Na^+$ -dependent excitation do resemble nicotinic receptors, whereas the receptors involved in the  $K^+$ -dependent ACh inhibition do not resemble any of the classical receptors.

Of the cholinomimetics, carbamylcholine is the only one that activates the three types of receptors. The receptors of the  $Na^+$ -dependent D-responses are activated by a variety of nicotinic agents: nicotine, tetramethylammonium, suxamethonium, and dimethylphenylpyperazinium. Decamethonium has slight effects (472). The H-receptors linked to  $Cl^-$ -dependent inhibition show similar responses to agonists but shows a selective sensitivity to PTMA (472). As stated, they differ in their sensitivity to HMT, which only blocks the D-receptors, whereas atropine seems to selectively block the D-receptors (472). Many of the cholinomimetics also have secondary blocking effects (472). The receptors of both the D-responses and the  $Cl^-$ -dependent H-responses may be also blocked by dihydro- $\beta$ -erythroidine, strychnine, and brucine (472).

The receptors involved in the  $K^+$ -dependent H-responses are completely different and show a rather unique pharmacology. They are activated or blocked by none of the agents that affect the other two types of receptors (469, 472). They are only activated by arecoline, which also has a secondary blocking effect (472), and blocked by methylxylocholine, TEA, and phenyltrimethylammonium (PTMA) (470, 472). Muscarine is not active in any of the three ACh receptors in *Aplysia* (472). It does not block the ACh responses of *Planorbis corneus*, which also show sensitivity only to nicotinic agents (892, 893, 962). Some neurons of *Helix* and *Navanax* may show responses to muscarine-like agents (559, 898). However, it is difficult to

TABLE 1. *ACh, DA, and 5-HT receptors in Aplysia and snail neurons*

Substance	Physiological Action	Ionic Permeability Change	Blocking Agents
ACh	D-response EPSP	Mainly $\text{Na}^+$ ( $\text{K}^+?$ $\text{Ca}^{++}?$ )	dTC, HMT, strychnine
	Slow H-response Slow IPSP	$\text{K}^+$	TEA, methylxylocholine
	Fast H-response Fast IPSP	$\text{Cl}^-$	dTC, strychnine
DA	Depolarization	Mainly $\text{Na}^+$ ( $\text{K}^+?$ $\text{Ca}^{++}?$ )	dTC, strychnine
	Hyperpolarization	$\text{K}^+$	Ergometrine
5-HT	A-response (depolarization)	Mainly $\text{Na}^+$ ( $\text{K}^+?$ $\text{Ca}^{++}?$ )	dTC, LSD 25, bufo-tenine
	B-response (hyperpolarization)	$\text{K}^+$	LSD 25, bufotenine
	C-response (hyperpolarization)	$\text{Cl}^-$	dTC, prostigmine, LSD 25

compare the results obtained in *Aplysia* with those reported in *Helix* (898–900) and *Planorbis* (892, 893, 962) since the applications of the agonists in the last two were done by perfusing the drugs onto spontaneously firing cells. This does not seem an appropriate condition to evaluate the actions of drugs on molluscan neurons.

Quantitative work on ACh receptors of molluscan neurons is still lacking.

E) LOCALIZATION AND DISTRIBUTION OF RECEPTORS: SINGLE AND MULTIRECEPTOR RESPONSES. There is now relatively good evidence that the somatic membrane of molluscan neurons is endowed with ACh receptors (322, 469, 472, 559, 783, 784, 826, 830). The physiological significance of the presence of receptors in regions devoid of synaptic contacts is obscure. There are indications that the receptors located on the axon nearest the synaptic region are perhaps more sensitive to ACh than the somatic ones (783, 830).

Neurons may be endowed with only a single type of ACh receptor or with multiple receptors (469, 472, 559, 894, 895). In the latter case multiphasic responses are recorded when ACh is applied iontophoretically and, correspondingly, stimulation of appropriate inputs or interneurons causes multiphasic postsynaptic potentials. In the medial pleural neurons of *Aplysia*, ACh application causes a biphasic response resulting from the coupling of a rapid  $\text{Cl}^-$ -dependent H-response and a slow  $\text{K}^+$ -dependent H-response (469, 470, 472). In the neuron L7 of the visceral ganglion another type of multireceptor response may be recorded consisting of a  $\text{Na}^+$ -dependent D-response coupled to an  $\text{Cl}^-$ -dependent inhibition (894, 895). Some neurons may even show ACh responses by combining the activation of the

three receptors described above (472). In *Navanax* buccal neurons show biphasic ACh responses combining  $\text{Na}^+$ -dependent depolarization with a  $\text{Cl}^-$ -dependent hyperpolarization (559).

In some of the cases of multicomponent responses in *Aplysia* (472) and *Navanax* (559) the displacement of the tip of an iontophoretic pipette along the surface of the neuron may evoke different responses at different points, showing the presence of different kinds of receptors either on the soma or on the axon. Biphasic responses combining the activation of the two receptors may be recorded by ejecting ACh on points intermediate between regions showing different responses or by passing large iontophoretic currents that eject larger concentrations of ACh, thus recruiting receptors from a large area (559).

In some cases (22, 472, 559) the multireceptor character of the ACh response is not straightforwardly apparent and only becomes manifest by pharmacological manipulations that block some of the receptors without affecting the others (see examples above).

F) CHOLINERGIC PSP'S: CHOLINERGIC INTERNEURONS MEDIATE OPPOSITE SYNAPTIC ACTIONS. The endogenous activity of molluscan and especially gastropod neurons is modulated by synaptic activity, inhibition and/or excitation (16, 130, 815, 817), which may be easily detected when recording intracellularly from the neuronal somata. Tauc (815–817) first described elementary IPSP and EPSP in gastropod ganglia and postulated their chemical character.

Neurons showing  $\text{Cl}^-$ -dependent H-responses to ACh also show unitary IPSP's that could be blocked by dTC and atropine, whereas neurons endowed with receptors involved in D-responses showed EPSP's that were gradually and reversibly blocked by dTC, atropine, and hexamethonium (828–830). The excitatory input of neurons showing the H-responses generally was not altered by cholinergic antagonists (see exceptions below). Equally, in general the inhibitory activity observed in many D-neurons was not affected by cholinergic blocking agents (311, 830). These data indicate that probably ACh may be the transmitter of excitation to the cells showing D-responses and of inhibition to the neurons showing H-responses. Tauc and Gerschenfeld (829) postulated the possibility that in gastropod ganglia the same interneuron releasing ACh through different endings could cause excitation of H-responding neurons and inhibition of the D-responding neurons. The existence of such an interneuron was strongly suggested by experiments of Strunwasser (787) and elegantly demonstrated by Kandel and his colleagues on *Aplysia californica* (448, 449, 451). The interneuron L10, located in the ventral face of the visceral ganglia, which has now been proven to be cholinergic (331), appears to make a likely monosynaptic contact with a series of neurons, among them neurons L3 and R15. L3 responds to ACh by an H-response, whereas R15 gives a D-response and shows an EPSP that may be blocked by dTC and hexamethonium (317). Intracellular stimulation of L10 evokes spikes and for each spike causes both an IPSP in the L3 neuron and an EPSP in the R15 neuron (449, 451). dTC blocks both PSP's without altering the spike of L10 (451).

Similar interneurons evoking opposite synaptic activity, probably also cholinergic in character through different branches, may also be observed in the buccal ganglia (299, 787) and in the pleural ganglia of *Aplysia* (474).

In all the described cases a parallelism of pharmacological properties was generally observed between the responses of the ACh extrasynaptic receptors and the responses of the synaptic receptors as determined by the effect of drugs on the post-synaptic potentials (451, 472, 499, 829, 830). The same is also true of the ionic basis for the cholinergic IPSP's and EPSP's. The IPSP's observed in neurons responding to ACh with a  $\text{Cl}^-$ -dependent H-response also were found to be due to an exclusive change of the membrane permeability to  $\text{Cl}^-$  ions (75, 135, 474, 499, 751, 752). The analysis of the ionic mechanism of the EPSP is complicated by the secondary effects of changes in  $[\text{Na}]_o$  on spike configuration and thus on transmitter release, especially when  $E_{\text{ACh}}$  cannot be directly observed but must be calculated by extrapolation. However, from the available data it would appear that both the EPSP's and the ACh D-responses (see above) involve mainly a change in membrane permeability to  $\text{Na}^+$  (75). The implications of this bimodal effect of interneurons on follower neurons for the integrative functions of molluscan CNS is beyond the scope of this review and has been extensively analyzed by Kandel and his colleagues (450, 912, 913, see 453). Apparently the same organizational feature is possibly true for the case of noncholinergic interneurons (453).

G) CHOLINERGIC MULTIPHASIC PSP'S. As described above, the medial pleural neurons of *Aplysia* show a multireceptor response to ACh combining a  $\text{Cl}^-$ -dependent rapid inhibition with a  $\text{K}^+$ -dependent slow inhibition (469, 470, 472). Kehoe (469) first observed in these neurons a biphasic IPSP when the tentacle was stimulated by water drops or when the tentacular nerves were electrically stimulated. This biphasic IPSP is composed of an early fast IPSP followed by a late slow IPSP. As with the rapid ACh H-response, the early IPSP may be blocked by dTC and other nicotinic antagonists, while the slow IPSP, in analogy to the slow H-response, is only blocked by agents like methylxylocholine or low concentrations of TEA (469, 470, 472). The reversal potentials of the early IPSP and the rapid H-response are both near  $-60$  mv. A reversal potential level of  $-80$  mv may be measured for both the late IPSP and the ACh slow H-response (469, 472, 474). After previous pharmacological isolation of each IPSP, Kehoe (469, 473) has shown that the early IPSP is  $\text{Cl}^-$ -dependent whereas the late slow IPSP is  $\text{K}^+$ -dependent (473). Again the similarities between extrasynaptic and synaptic responses are well demonstrated and ACh is very probably the transmitter responsible for both phases. Further work (471, 474) allowed the identification of an interneuron in the pleural ganglion that mediates both phases of the IPSP. Intracellular injection of TEA into this interneuron increases the duration of its action potentials and leads to an increase of the amplitude of both the fast and slow components of the IPSP (471, 474). This is an excellent demonstration of the origin of both phases from the firing of only one interneuron. This same interneuron may also cause an opposite effect, an EPSP, in the anterior pleural neurons that shows  $\text{Na}^+$ -dependent responses when tested with ACh applied onto their membrane (474).

Other types of synaptic responses that would correspond to a multireceptor activation on a single neuron have been described by Wachtel and Kandel (894, 895). In the visceral ganglion of *Aplysia californica* intracellular stimulation of the interneuron L10 at low frequencies (less than 1/sec) induces almost synchronous repetitive EPSP's in neuron L7. When the frequency of L10 is increased (over 6–10/sec)

the EPSP's in L7 diminish in amplitude and are then replaced by IPSP's, following almost synchronously and summatting to form a hyperpolarizing wave (894, 895).

Following at relatively high rates, together with the similarity of the latencies of the two IPSP's and EPSP's, provoked in L7 by the firing of L10 indicates that both phases are probably initiated by the same interneuron (895). However, the present evidence does not eliminate the existence of a chain of one tightly interposed interneuron. The pharmacological evidence, on the other hand, supports the cholinergic character of both types of synaptic potentials. ACh application by iontophoresis to the somatic membrane of neuron L7 also causes a biphasic response composed of a  $\text{Na}^+$ -dependent D-response, followed by a  $\text{Cl}^-$ -dependent H-response (895). dTC was effective in blocking both H- and D-responses as well as both EPSP's and IPSP's initiated by stimulation of interneuron L10 (895). dTC does not block other EPSP appearing "spontaneously" in neuron L7 (895). Pharmacological tools like HMT were not further used to distinguish between the two different ACh receptors involved in these biphasic responses. The reversal potentials of the EPSP and IPSP evoked on neuron L7 by the firing of interneuron L10 are respectively equal to the  $E_{\text{ACh}}$  of the D- and the H-responses elicited by ACh on the extra-synaptic membrane of L7 (75). Both the EPSP's and the D-responses appear to be mainly  $\text{Na}^+$ -dependent, whereas both the IPSP's and the H-responses depend on  $\text{Cl}^-$  (75).

The mechanism of the conversion from EPSP to IPSP by changes in interneuron frequency may be interpreted in different ways. Wachtel and Kandel (895) propose that a difference in the desensitization properties of both types of receptors involved could explain the conversion. If D-receptors would desensitize more readily than the H-receptors, the ACh concentration increase in the cleft due to increased interneuron frequency would favor a predominance of the responses of the H-receptors. A simultaneous sensitization of the H-receptors would even increase the rate of this process (60). An alternative explanation could lay in a pre-synaptic mechanism such as the blocking by high frequency of some axon terminal branches facing D-receptors, whereas the transmitter is released by branches facing H-receptors. No effort has yet been made to use hexamethonium to separate the two components to analyze their behavior independently, with respect to interneuron frequency.

H) ACH AND THE ELECTROGENIC  $\text{Na}^+/\text{K}^+$  PUMP. It has already been mentioned that an electrogenic  $\text{Na}^+/\text{K}^+$  pump may somehow participate in the regulation of the membrane potential of molluscan neurons. Recent papers have postulated that ACh released from synaptic endings may be able to produce postsynaptic effects, not only through changes in the ionic permeability of the postsynaptic membrane but also by causing the activation of the electrogenic  $\text{Na}^+/\text{K}^+$  pump. According to this view ACh would accelerate the electrogenic pump, which would extrude  $\text{Na}^+$  faster than  $\text{K}^+$  is moved inward, thus causing a hyperpolarization resulting from an asymmetric partition of charge.

The neuron L2 of the visceral ganglion of *Aplysia californica* responds to a single spike in the interneuron L10 by showing a two-wave synaptic inhibition composed by an "early" IPSP and a late IPSP (693). The early IPSP shows a reversal poten-

tial of  $-65$  mv and is due to an increased permeability to  $\text{Cl}^-$ . It could be blocked by dTC like the other IPSP. The late IPSP is generally not reversed by increasing the membrane potential of the neuron L2 even when the somatic membrane is driven to levels much more negative than  $E_K$ . No conductance changes could be detected during the late IPSP by passing square pulses and recording from the soma (693).

The late IPSP may apparently be suppressed by agents known to block the  $\text{Na}^+/\text{K}^+$  pump such as ouabain, cooling to  $7\text{--}10$  C, or a very prolonged washing with  $\text{K}^+$ -free saline (693). ACh applications onto the soma would mimic the effects of synaptic activation. Pinsker and Kandel (693) concluded that ACh on neuron L2 caused an early change of  $\text{Cl}^-$  permeability followed by an activation of the  $\text{Na}^+/\text{K}^+$  electrogenic pump.

Recent experiments on the same neuron (L2) by Kehoe and Ascher (475) lead to a completely different interpretation. These authors note first that the  $\text{K}$ -dependent slow IPSP forming part of biphasic responses of the medial pleural neurons (see above) is affected by ouabain in way similar to the late IPSP of the neuron L2 of visceral ganglion (474, 475). If, after blocking the early  $\text{Cl}^-$ -dependent IPSP with dTC, ouabain is applied while keeping the neuron at a steady  $E_m$  of  $-70$ , the hyperpolarization disappears. However, when  $E_m$  is driven to other levels, the slow IPSP reappears because ouabain was actually causing a shift of the  $E_{\text{IPSP}} = E_K$ . Thus a hyperpolarization reappears when the membrane potential is set at  $-50$  mv or it reverses when  $E_m$  is driven beyond  $-70$  mv. This shift of  $E_{\text{IPSP}}(E_K)$  is due to a change in the  $\text{K}^+$  gradient resulting from accumulation of  $\text{K}^+$  in the extracellular clefts, after the blocking of the  $\text{Na}^+/\text{K}^+$  pump (474, 475, 737). Moreover, cooling of the pleural ganglion to  $7\text{--}10$  C blocks both the slow ACh response and the slow IPSP.

The biphasic inhibition of neuron L2 of the visceral ganglion is similar to that of the medial pleural neurons (475). After blocking the early IPSP with dTC, the late IPSP can be seen to be composed of a multiwave hyperpolarization; a large part reverses at  $-90$  mv and it is also  $\text{K}^+$ -dependent. The behavior of this wave when ouabain is perfused is similar to the behavior of the slow IPSP in the medial pleural neurons (475). The remaining components, which do not reverse, correspond probably to an electrotonic biphasic potential similar to those already described in *Aplysia* neurons (475, 824, 910, 911). It must then be concluded that there are no grounds as yet in *Aplysia* ganglia to postulate a cholinergic activation of the electrogenic pump.

Such a synaptic mechanism also has been claimed in the case of some synaptic inhibition activities in *Helix* (481), which are not sensitive to changes in the  $\text{Cl}^-$  ionic gradient, are diminished when  $\text{Na}^+$  is removed from the external medium, and are blocked by ouabain. The possibility of ACh acting through a change in membrane  $\text{K}^+$  conductance is not challenged by these findings nor the possibility that the effect of ouabain was to shift  $E_K$ .

Summarizing, it may be said that ACh is very probably a transmitter in the gastropod CNS, which when released from different branches of cholinergic interneurons may convey different physiological actions according to the presence of one

or more specific receptors in the postsynaptic neurons. The activation of each receptor is linked to a selective change in the membrane permeability to an ion species, and the fluxes thus caused may produce excitation or inhibition or different combinations of both processes.

5) *Dopamine*. The biogenic amines DA and 5-HT may have some transmitter function in molluscan CNS and at the periphery (see sect. vi). The evidence is rather indirect and mainly circumstantial. In no case has it reached the persuasiveness of the experimental proofs about the role of ACh.

Dopamine is the main catecholamine present in molluscan central ganglia. Since early work (237, 670) the absence of epinephrine and NE from molluscan nervous tissues was remarked. However, recently some NE has been detected in *Helix* brain as well as in some of the metabolites resulting from its inactivation by MAO in other CNS (665).

Dahl et al. (170) were the first to report that DA was present in *Helix pomatia* ganglia in a concentration of 7.25 µg/g. Sweeney (795) showed the abundance of DA in a survey of the central ganglia of 10 species of gastropods and pelecypods. For instance, a maximum content of DA of 261 µg/g tissue is observed in *Mercenaria* central ganglia (795). Since then many investigators have confirmed the presence of DA in amounts varying from a few micrograms to 20–30 µg in *Helix* ganglia (116, 493, 666) and *Aplysia* (124). In *Aplysia* some ganglia (e.g. pleural ganglion) are devoid of DA, whereas others (e.g. pedal ganglia) are rich in the amine (24 µg/g tissue) (124). It is highly probable that in great part the amine may be inside neurons. Histochemical studies with the fluorescence technique of Falck and Hillarp (see 245) demonstrate many green fluorescent neurons and axons interpreted as likely to contain DA. This finding has been repeatedly reported in different molluscan species: *Buccinum undatum* (170), *Helix pomatia* (170, 171), *Tritonia diomedea* (739), *Anodonta cygnea* (966), *Helix aspersa* (761), *Limnaea stagnalis* (740), *Sphaerium sulcatum* (796), *Strophocheilus oblongus* (434), *Planorbis corneus* (581), and *Limax maximus* (667).

In all these observations, fluorescent varicose fibers of various lengths and diameters have been described in ganglionic neuropiles and peripheral nerves. Extrapolating from vertebrate fluorescence studies, it may be supposed that the varicosities correspond to synaptic endings. Reserpine causes a fall in the DA content of the ganglia and a fading of the green fluorescence of nerves and axons (157, 170, 171, 493, 760, 966). A relation between dense-core granulated synaptic vesicles and DA has been postulated on the basis of alterations in their size and content under the effect of reserpine (633, 967).

There are very few data about the mechanism of synthesis of DA in molluscan neurons. Homogenates of *Helix pomatia* nervous tissue decarboxylate dopa, converting it to DA (116). Normally dopa circulates in snail blood (493) but it is not clear if it is the physiological precursor of dopamine. Although increases in the DA content of ganglia after dopa injection to the whole animal have been reported (493), the evidence regarding the mechanism of DA synthesis is still incomplete.

The mechanism of DA inactivation in molluscan CNS is also unclear. A monoamine oxidase has been detected in molluscan nervous and muscular tissues (77,

116), but in *Helix* ganglia this MAO is not able to oxidize dopa. Inhibitors of MAO in vertebrate tissues were shown to be ineffective in changing DA concentration in *Helix* CNS (116, 493). Recently, Cardot (119) found that cytochrome oxidase was able to oxidize DA in *Helix* ganglia. However, Osborne and Cottrell (665) have detected in *Helix* brain the presence of both homovanillic acid and of 3,4-dihydrophenylacetic acid. These metabolites result in vertebrate brain from the inactivation of DA by MAO respectively before and after the attack of DA by catechol-*o*-methyltransferase (COMT). On the other hand, McCaman and Dewhurst (588) found a ubiquitous distribution of COMT in *Aplysia* neurons not related to the amine content of the neurons.

Another mechanism of DA inactivation could be its uptake by tissue elements.  $^3\text{H}$ -DA uptake has been observed in *Aplysia* ganglia, but histoautoradiography shows that it is in great part bound to the tissue envelopes (23). On the other hand, Carpenter et al. (124) claim that a part of the uptake of DA by *Aplysia* ganglia may be blocked by desipramine. However, the possibility of an artifact cannot be ruled out since the pleural ganglia, which does not contain DA, also takes up the amine. Moreover, in *Helix*, neurons that are yellow fluorescent because they probably contain 5-HT may also take up DA (760). The entire question requires further study.

A) DA RESPONSES AND THEIR PHYSIOLOGICAL SIGNIFICANCE. Perfusion of DA onto *Helix* and *Aplysia* ganglia may cause opposite and different effects on neurons: some of them become hyperpolarized and inhibited (311, 326, 334, 500, 902, 947), whereas others are depolarized and excited (326, 334). Ascher (20, 21) has performed a very thorough study of dopamine receptors in the pleural ganglion of *Aplysia californica* by using the iontophoretic application technique. The anterior pleural cells (see 472) are depolarized and excited by dopamine pulses, whereas the medial pleural neurons are generally hyperpolarized (21). On the other hand, the posterior pleural cells show biphasic responses due to multireceptor activation (21). When DA is applied onto the soma, the responses are seldom obtained; the receptors therefore may be located on the axon, the responses improving when the iontophoretic pipette is placed on the axon hillock or beyond it, sometimes far away from the soma (21).

The depolarizing responses can be blocked by dTC and strychnine (20, 21). It is still difficult to know exactly the character of this blocking action, since both drugs also block some ACh receptors (see above) and dTC may also block 5-HT receptors (see below). The effects of DA do not seem to be mediated by an ACh release from synapses since HMT does not affect the DA response. However, as will be seen, neither dTC or strychnine blocks the hyperpolarizing DA responses, and therefore they are good pharmacological tools to analyze biphasic responses (21). There are indications that DA depolarization is mainly due to a change in membrane permeability to  $\text{Na}^+$  (Ascher, personal communication). Seven norepinephrine antagonists (among them phentolamine, tolazoline, phenoxybenzamine, etc.) seem to act more on the depolarizing DA responses than on the hyperpolarizing ones, but also show unspecific effects altering the resting membrane resistance (21). The receptors mediating DA depolarizations are desensitized readily by protracted

perfusion of the drug or its repeated iontophoretic application. These receptors are more profoundly desensitized than the ones involved in the DA hyperpolarization (21).

The DA hyperpolarizing responses of the medial pleural neurons can show varied reversal potentials. In some cases they are reversed at  $-80$  mv and the level shifts with alterations in  $[K^+]$ , as predicted by the Nernst equation (21). In other cases  $E_{DA}$  is found to lay at more depolarized values than  $-80$  mv. In these cases, when dTC is perfused onto the preparation  $E_{DA}$  equal to  $-80$  mv and dependent on  $K^+$  may be demonstrated. This shows that the intermediate reversal potential value was due to the simultaneous activation of another receptor involved in DA depolarization (21). In a third group of neurons DA hyperpolarizations could not be reversed and this may be demonstrated as being due to difficulties to drive the  $E_m$  of the actual receptor region to a level beyond  $-80$  mv by passing inward current into the soma (see 474). These responses, which may behave under the effects of ouabain and cooling similarly to the  $K^+$ -dependent H-response to ACh, are also due to a change of membrane permeability to  $K^+$  (21).

Hyperpolarizing responses to DA in *Helix* and *Aplysia* are selectively blocked in rather low concentrations by ergot alkaloids, ergotoxine, ergometrine, and ergotamine. In *Helix* they may also be blocked by rogitine, dibenlyline, and yohimbine, i.e. they resemble adrenergic  $\alpha$ -receptors of vertebrates (21, 902). Ergot alkaloids in concentrations higher than  $10^{-5}$  g/ml may act as agonists and cause neuron hyperpolarization by themselves (21).

The use of dTC and ergot derivatives permits a pharmacological "dissection" of the biphasic responses to DA of the posterior pleural neurons of *Aplysia*. They consist of an excitatory wave followed by an hyperpolarization, and selective blocking proves the coexistence on the same neuron membrane of both types of DA receptors (21). Table 1 summarizes the pharmacology of DA receptors.

Ergot derivatives recently have been claimed to block some synaptic inputs in *Helix* neurons (486, 901).  $K^+$ -dependent noncholinergic IPSP's recorded in *Helix* neurons (311, 319) were shown to diminish in amplitude when the neurons were bathed with ergometrine (901). This also seems to happen (486, 901) to the "inhibition of long duration" (ILD), a long-lasting hyperpolarizing response that appears in some neurons after orthodromic stimulation, sometimes preceded by an EPSP (818, 819; see 824). But, as Ascher (21) observes, the doses of ergometrine used for blocking the synaptic potentials are almost 100 times higher than those needed for blocking DA responses. Such concentrations not only diminish the amplitudes of IPSP and the inhibitions of long duration, but also affect other known cholinergic inputs (21). On these grounds it would appear premature to accept that DA mediates those inhibitory synaptic actions.

6) *Serotonin*. 5-HT is one of the biogenic amines found in the central nervous system and the periphery (e.g. heart muscle) in molluscs. Welsh and Moorhead (921) in a survey on 5-HT contents in the CNS of different invertebrates phyla found that molluscs were one of the richest in 5-HT, the highest concentration ( $40$   $\mu$ g/g tissue) being observed in *Venus mercenaria* (see also 919, 922). In general the 5-HT content of ganglia is lower, about  $2$ - $6$  mg/g tissue in *Helix* (121, 483), in

*Aplysia* (124), and in a series of marine snails (617). 5-HT also is abundant in cephalopod nervous tissue (271, 728, 921).

The distribution of the amine is not uniform for a given nervous system. In *Aplysia*, for instance, the buccal ganglia or the bag-cell region of the visceral ganglia do not contain detectable 5-HT (124).

There is now little doubt that an important fraction of the amine is most probably localized in the neurons. Yellow fluorescent neurons are observed with the Hillarp-Falck technique (245) and interpreted as containing 5-HT in many species: *Buccinum undatum* (170), *Helix pomatia* (170, 171, 666), *Anodonta cygnea* (966), *Lymnaea stagnalis* (740), *Helix aspersa* (761), *Sphaerium sulcatum* (796), *Planorbis corneus* (581), *Limax maximus* (667), *Strophocheilus oblongus* (434).

Some of these papers gather spectrofluorometric evidence relating the neuronal yellow fluorescence to the presence of 5-HT. Less agreement exists about the localization of 5-HT at the level of the neuropile.

Reserpine depletes 5-HT from the ganglia (616, 617) and causes fading of the yellow fluorescence from neurons (171). Reserpine may also affect the rate of 5-HTP decarboxylation of the ganglia (617).

Cottrell and Osborne (158, 165, 667) have identified a giant neuron in the cerebral ganglia of *Helix* and *Limax* that possibly is serotonergic. This cell was first described as the metacerebral giant neuron (MCGN) by Kunze (535) and its electrophysiological and input properties were already described in *Helix* by Kandel and Tauc (454, 455). The MCGN shows bright yellow fluorescence with the Hillarp-Falck technique and extracts obtained from many isolated MCGN mimic well the effect of 5-HT on molluscan heart (165). Reserpine reduces the fluorescence of the MCGN (165, 667).

Axons located in peripheral nerves, such as the visceral nerve in *Aplysia* and *Helix*, also show a yellow fluorescence when studied with the Hillarp-Falck technique (668, 831). Ligatured peripheral axons may show accumulation of yellow fluorescent material in one of the sides of the ligature, suggesting a transport of 5-HT along nerve fibers (668).

No direct evidence is available about the presence of 5-HT in molluscan nerve endings. In vertebrate nervous system 5-HT has been found bound to particulate elements (vesicles) in the synaptosome subcellular fraction (576, 604, 963). Cell fractionation applied to molluscan CNS has not given any clear result; 5-HT is claimed to be bound to endoplasmic reticulum membranes (970) or to unidentified particles different from those that bind ACh (155, 163). On the other hand, cytochemical ultrastructural methods supposed to detect 5-HT, such as the Wood technique (946), applied to *Amisodoris* central neurons and to the MCGN of *Limax* (165, 446) suggest that 5-HT may be associated with dense-core granulated vesicles 900–1200 Å in diameter. Apparently during some seasons 5-HT would also be bound to lysosomes (165).

Nervous tissue homogenates of different molluscan species may decarboxylate 5-HTP into 5-HT (386, 617, 920). The MCGN of *Limax* seems to be able to synthesize 5-HT from 5-HTP (167). Aromatic amino acid decarboxylase involvement in this metabolic step, as in the synthesis of dopamine, is not specific. In *Aplysia* the

aromatic amino acid decarboxylase is ubiquitous throughout the CNS: all the individual neurons assayed were found to contain the enzyme (916a). No direct information on the existence in molluscan ganglia of tryptophan hydroxylase, the enzyme synthesizing 5-HTP from tryptophan, is available. The only claim about the existence of this enzyme in molluscan CNS is that Cardot (120) recently reported showing that *p*-chlorophenylalanine, an inhibitor of the enzyme in other nervous tissues, depletes the 5-HT content of central ganglia of *Helix pomatia*.

Another point deserving further study is the problem of 5-HT inactivation either in the intraneuronal pools or in the hypothetical release into the synaptic clefts. MAO has been demonstrated in *Helix* ganglia but does not seem to be active on a 5-HT substrate (115, 117), whereas it may act on substrates such as tryptamine (118) (see below for further discussion about the possible method of inactivation of 5-HT if released into the synaptic cleft).

Endogenous 5-HT may be released by nerve stimulation. An indolamine that is recognized by bioassay or spectrofluorometry as 5-HT may be recovered from heart perfusates after electrical stimulation of cardioaccelerator nerves of *Mercenaria* or *Helix* (565, 781; see 164). Endogenous 5-HT may also be recovered from the saline bathing a snail CNS preparation. The quantity of 5-HT recovered from an unstimulated CNS after 3 hr is at the level of the background values of the fluorimetric technique used for assay, but nerve stimulation at frequencies of 3–7/sec during the same period increases at least 10 times the amine recovered from the bath (cf. 324). The case seems different for exogenous labeled 5-HT taken up previously by *Aplysia* ganglia (see below), which may be also released into the bathing saline by electrical field stimulation (134). This release may be an artifact, since histoautoradiography of the ganglia shows that a great part of the 5-HT taken up is located in the envelopes of the ganglia (see 23) and field stimulation of the isolated envelopes may also release 5-HT into the bathing solution (23).

a) 5-HT RECEPTORS. Application of 5-HT by perfusion to *Aplysia* or snail ganglia results in the excitation of some neurons (321, 322, 334) and the inhibition of other neurons (325, 334). In snail ganglia (*Helix* and *Cryptomphallus*) these pharmacological actions of 5-HT iontophoretically applied appear to result from the activation of three different types of receptors that have been named A-, B-, and C-receptors (315, 316, 320a).

5-HT A-receptors are present on the axon hillock and beyond it in neurons that generally exhibit an inhibition of long duration when stimulated orthodromically (CILDA cells, see 322). Their activation results in a rather long-lasting depolarization and excitation of the cells, probably due to a change in the membrane permeability mainly to Na<sup>+</sup> (324, 784). As with the receptors involved in DA excitatory responses, they may be blocked by dTC but can be easily differentiated from neighboring ACh receptors, whose activation also causes a Na<sup>+</sup>-dependent D-response, by using hexamethonium, which blocks the ACh receptors exclusively. On the other hand, LSD 25, tryptamine, bufotenine, and 5-HT itself block the 5-HT A-receptors without affecting the ACh receptors (320a, 323, 324, 784). Moreover, anticholinesterase drugs prolong the effects of ACh but do not affect the 5-HT A-responses. 5-HT A-receptors are greatly desensitized after repetitive 5-HT appli-

cation (321, 322). Monoamine oxidase inhibitors block the 5-HT A-receptors (322). Atropine and morphine may also block the A-receptors (322).

5-HT B-receptors are quite different: their activation brings out in some identified cells of snail CNS hyperpolarization and inhibition of the neuron that is frequently difficult to reverse by hyperpolarizing the neuron, due both to the location of the receptors far away from the soma and to anomalous rectification phenomena (315). When  $E_{5\text{-HT}}$  can be measured it is around  $-75$  to  $-80$  mv, shifting with changes in  $K_o$ , as may be predicted from the Nernst equation (315, 320a). Cholinergic blocking agents do not affect the 5-HT B-receptors, which also may be blocked by LSD 25, bufotenine, tryptamine, and 5-HT itself (315, 316, 320a). Desensitization of these B-receptors is less marked than those of the A-receptors.

5-HT C-receptors also appear in a very restricted number of identified snail central neurons and their activation also causes inhibition of the neuron. This hyperpolarization reverses at  $-55$  or  $-60$  mv and is due to a  $\text{Cl}^-$  influx caused by an increase of the neural  $\text{Cl}^-$  permeability (315). Again, cholinergic drugs seem to act on this receptors as on the neighboring ACh H-receptors, which are also involved in a  $\text{Cl}^-$ -dependent inhibition (315-317). There has not yet been found any pharmacological way to distinguish between the 5-HT C-receptors and the neighboring ACh receptors (316, 317); dTC blocks both of them as does atropine, eserine, or prostigmine. Bufotenine does not block them (320a). The possibility of an indirect action of 5-HT causing the release of ACh from cholinergic endings may be ruled out, since removal of  $\text{Ca}^{++}$  plus a fivefold increase of  $\text{Mg}^{++}$  concentration in the presence of 5-10 mm of EGTA in the medium (conditions that cause complete blockade of all transmitter release) are not capable of suppressing the 5-HT effects (316, 317). Moreover, crossed desensitization between ACh and 5-HT is observed in the neurons endowed with 5-HT C-receptors (316, 317). This remains unexplained. Biphasic 5-HT responses arising from the activation of two receptors located on the same neuronal membrane are observed (314, 316, 320a). Table 1 summarizes the properties of the three types of receptors.

b) 5-HT AND SYNAPTIC INPUTS. There is only circumstantial evidence that 5-HT A-receptors may be involved in synaptic activation of the neurons endowed with them. Such neurons may show two different types of EPSP when stimulated orthodromically: fast EPSP (duration 200-300 msec) and slow EPSP (duration more than 800 msec) (324). These EPSP's are unitary (appearing in an all-or-none way by changing stimulation parameters), but in some cases fast monosynaptic EPSP's appear spontaneously due to the discharge of an interneuron. Hexamethonium blocks both the spontaneous or evoked fast EPSP without altering the slow one, whereas LSD 25, tryptamine, and 5-HT block the slow EPSP without affecting the fast EPSP (324). This blockade is gradual, but since the slow EPSP's were not monosynaptic it is difficult to discard a presynaptic effect of the blocking agents (324). It is interesting here to see, as was also observed in *Aplysia* (318), that a neuron may have excitatory inputs mediated by two different transmitters—ACh and some other noncholinergic transmitter. Further data are required to

accept these findings as conclusive evidence for a synaptic transmitter role of 5-HT.

Recently Cottrell (158, 159, 161) found that the MCGN, which possibly is a serotonergic cell (see above), sends out branches to the buccal ganglion. Intracellular stimulation of the MCGN results in low-amplitude EPSP's in a group of buccal neurons (158, 161). This EPSP discharge follows relatively high frequencies of MCGN discharge, which is claimed to suggest a direct and monosynaptic connection between these neurons (158, 161). The buccal neurons are depolarized by 5-HT and LSD 25 blocks the EPSP, whereas methysergide potentiates it (158, 159). Imipramine, supposed to block 5-HT uptake, seems to potentiate the effects of stimulation (160) (see below). The very small amplitude of the EPSP's recorded in the buccal neuron makes it difficult to evaluate these pharmacological results. Moreover, a better proof of the monosynaptic link between the MCGN and the buccal neuron is necessary. However, such a line of research may be promising for the question of the synaptic role of 5-HT.

c) INACTIVATION OF 5-HT. If it is supposed that 5-HT is a transmitter and as such is released by action potentials into the synaptic cleft, it needs to be inactivated to account for the limited duration of the synaptic currents. We have already commented on the lack of information about the existence of a metabolic pathway to dispose of 5-HT, since MAO does not seem to be active on a 5-HT substrate (117, 118). Other mechanisms have been proposed: diffusion, desensitization, or uptake (see 134, 324). There is now some evidence about the uptake of 5-HT by molluscan nervous tissues using electron-microscopic autoradiography. Taxi and Gautron (831) observed that [<sup>3</sup>H]-5-HT was incorporated in nerve endings in *Aplysia* heart. These nerve endings generally contained clear synaptic vesicles of 300–600 Å. At variance with cardiac auricle tissue, which may accumulate almost 10 times the quantity of 5-HT available in the bath, *Aplysia* ganglia exposed to tritiated 5-HT will not accumulate more than 3 times the external concentration (124, 134). Auricle uptake is depressed when Na<sup>+</sup> is removed from the environment and/or ouabain or desipramine is added (124). Changes in [Na]<sub>o</sub> do not affect the 5-HT uptake by the ganglia, and desipramine or ouabain only partially depresses the uptake. This confirms previous suggestions (23) that an important part of the uptake is nonspecific. In conclusion, the 5-HT uptake by cardiac nerves shows that such a mechanism exists and could be important in the physiology of serotonergic endings. However, its existence has not been confirmed yet at the CNS.

On the basis of the evidence summarized here, it may be said that there is interesting evidence in favor of a role for 5-HT as a synaptic transmitter in molluscan CNS, but this function needs to be fully proven.

7) *Amino acids.* Of the amino acids known to participate in synaptic transmission in other nervous system, only glutamate has been postulated as a transmitter in molluscan nervous system (see below).

Glutamic acid is normally a component of molluscan blood and nervous tissue. Nervous ganglia of *Helix* and *Eledone* incorporate [<sup>U-14</sup>C]glucose mainly into alanine, glutamate, and glutamine (91, 492). [<sup>U-14</sup>C]glutamate is mainly incorporated into glutamine (91, 154, 378).

In gastropod and cephalopod nervous tissue neither incorporation of label from [ $^{14}\text{C}$ ]glutamate into GABA nor the presence of glutamic acid decarboxylase could be detected (91, 154, 718). However, recently GABA has been detected in low amounts in *Helix* ganglia (664a).

Kerkut et al. (487) have reported release of glutamate from a molluscan ganglion-nerve-muscle preparation by nerve stimulation, but the amounts of glutamate recovered in the perfusate seem extremely large and incompatible even with the highest imaginable concentrations of glutamate in nerve or muscle (see 520). Moreover, Kerkut et al. (495) devised an *in vitro* preparation where the snail central ganglia remained connected by nerves to pharynx muscles, the ganglia and the muscle being placed in different compartments of a chamber. In this preparation they (495) claimed the existence of a transport of labeled glutamate incorporated by the ganglion and transported in the direction of the muscle, where it could be released by electrical nerve stimulation. Glutamate would thus travel the 1-cm distance from the ganglia to the muscle in 20 min, whereas the reverse would take 16–24 hr (495). Electrical stimulation would be able to release at the muscle ending 20–70% of the isotope bathing the nervous ganglia in 45 min (495). These results are difficult to believe since the isotope might also be metabolized by the nervous tissue (91, 378) and it may also be supposed that the number of neurons involved in the innervation of the pharynx muscle are only a small part of the ganglionic periesophageal ring (see 520).

Electrophysiological experiments with glutamate show that when applied to gastropod neurons it changes the membrane conductance and the responses observed are also dual: some neurons become excited and others become inhibited (320, 660, 741). Using iontophorctic applications, three probable receptors may be invoked in these actions since glutamate in different neurons may change the permeability either to  $\text{Na}^+$  or to  $\text{K}^+$  or to  $\text{Cl}^-$  (660; C. Lowagie and H. M. Gerschenfeld, unpublished observations). Biphasic effects of glutamate, probably due to the activation of two different receptors, may also be observed (C. Lowagie and H. M. Gerschenfeld, unpublished observations). Glutamate receptors generally show marked desensitization (320). Unfortunately specific glutamate receptor blocking agents are not available to allow discrimination between different types of glutamate receptors and to study their possible involvement in synaptic regions. It is also possible that these effects of glutamate have no physiological significance.

The possibility that GABA may play a role as a transmitter in the snail is very remote on the basis of present knowledge, particularly since the enzyme involved in its synthesis from glutamate in molluscan nervous tissue could not be detected (see above). However, GABA applied by perfusion or iontophoresis onto the snail neuron may produce inhibitory or excitatory effects (320), and GABA receptors, such as those of vertebrate CNS, may be blocked by picrotoxin or bicuculline (897).

#### B. Squid Giant Synapses

The giant synapses of the stellate ganglia of the squid (SGS) may be considered a gift of nature to synaptologists, since the size of the pre- and postsynaptic compo-

nents ( $\sim 100 \mu$  in diameter) allows implantation of microelectrodes at both the pre- and postsynaptic fibers. In spite of the lack of information about the identity of the transmitter involved, there is a sufficient body of data regarding the mechanisms of transmitter release at these synapses that when combined with information obtained from neuromuscular junctions constitute our main knowledge on the function of the nerve endings (see 412, 460).

1) *Structural background.* The giant fiber system of squids was rediscovered by J. Z. Young (956–958; see 961). It was previously studied by Williams (938), but he interpreted the whole system as originating from a single cell body located in the palliovisceral ganglion whose endings innervated the mantle muscles after a long and complex passage through the nervous system. Young (see 961) demonstrated that the system actually consists of three neurons and two synaptic relays. The first-order giant cells are the multipolar ones that Williams observed located on each side, at the magnocellular nucleus of the palliovisceral ganglia. The first-order axons arise from these neurons and each one crosses with the contralateral one, forming a chiasma, where they “fuse” by establishing tight junctions (951; see 582). Beyond this fusion the first-order axons branch and end, forming contacts with the second-order giant neurons. These are unipolar cells (seven or more) located in the posterior part of the palliovisceral lobes. Each sends two axons (one principal and one accessory) through the pallial connective to the stellate ganglia. In *Loligo* the main giant fiber of second-order neurons enters the stellate ganglion and divides in about 10 smaller branches. Each branch ends blindly, contacting the third-order axons. The third-order axons arise from the fusion of the axons of many unipolar neurons located in the stellate ganglion. The largest of the contacts between the second- and third-order axons is the *distal giant synapse*, which is formed by the second-order main axon and the largest third-order fibers, well known to neurophysiologists as the giant axon of the squid. The accessory second-order axon makes a series of contacts with the third-order fibers, and these are called the *proximal synapses*. The postsynaptic giant axons follow the stellar nerves (951; see also, 93, 611) and innervate the mantle.

An early ultrastructural study of the giant synapses was done by Robertson (721). Hama (358) and Castejon and Villegas (126) described more recently the ultrastructure of the giant synapse in the squids *Doryteuthis bleekeri* and *Sepioteuthis sepioides*. In the first animal many processes of the postsynaptic fiber (third-order fiber) actually penetrate the common sheath that separates them from the presynaptic fibers (second-order axons), making synaptic contact with the latter (358). A synaptic cleft of 200–300 Å separates the apposed surfaces of both axons and the membranes participating in the contact appear more opaque to electrons than the nonsynaptic axon membranes. On the presynaptic side typical organelles seen in chemical synapses are observed, such as mitochondria and clumps of electron-lucent synaptic vesicles 500–700 Å in diameter attached at some points to the inner face of the membrane (358).

In the squid *Sepioteuthis* the picture is somewhat different; here the afferent presynaptic axons send thin processes to make contact with the efferent postsynaptic axons. However, the structure of both proximal and distal synapses is the same

(126). Another important difference of the synapses of *Sepiotheuthis* from those of *Dorytheupis* is that two types of synaptic vesicles may be visualized in the endings of the latter: electron-lucent synaptic vesicles 500–700 Å and granulated vesicles that may attain diameters of 800–900 Å (126).

2) *Demonstration of chemical transmission.* The one-to-one character of transmission from second-order to third-order axons (100, 959) and the measurement of a synaptic delay (101) led to early interpretation in favor of a similarity of the transmission process to the vertebrate neuromuscular junction. Other indications in the same sense were the one-way functioning of the synapses, the recording of a synaptic potential similar to the EPP (101), and the fatigue of the synapse by repetitive stimulation. Subsequent work simultaneously impaling both pre- and postsynaptic axons with micropipettes resulted in definitive evidence for the chemical character of the transmission (93, 94, 104, 351, 611, 800).

The discharge of a presynaptic spike is followed after a short delay by the firing of a postsynaptic spike. Either by repetitive stimulation at high frequency or by polarizing the postsynaptic membrane, an excitatory synaptic potential not reaching threshold may be recorded. The measures of the synaptic delay were different according to various authors, the largest recorded being up to 1–2 msec (104). Miledi and Slater (611), using extracellular recording from a microelectrode critically located near the endings, measured a synaptic delay of 0.5–0.8 msec at 15°C, very similar to that recorded at the muscle end plate (461). The synaptic delay has a  $Q_{10}$  of 3 in both junctions (462, 557).

The existence of any electrical coupling between pre- and postsynaptic axons has been completely disproved. Subthreshold depolarizing pulses that do not reach the firing level of a presynaptic spike could not produce any potential variation in the postsynaptic membrane: in normal conditions only the discharge of the prefiber action potential results in an EPSP and a postsynaptic spike (104, 351). Hyperpolarizing the presynaptic axon by passing intracellular square inward current pulses does not produce any change of the membrane potential of the postfiber (351). However, the situation could be similar to that observed at electrical rectifying synapses in other invertebrates, where rectification provides a one-way mechanism of neuron activation (294, 644). Nevertheless, this is not the case in the squid giant synapse, since neither an antidromic postsynaptic spike nor a hyperpolarizing square inward current passed through the postsynaptic membrane has any influence on the presynaptic membrane potential (351). Moreover, using the square-pulse technique one may demonstrate that a change of the postsynaptic membrane conductance is involved in the generation of the EPSP (351). Takeuchi and Takeuchi (800) were also able to show with voltage-clamp techniques that there is a linear relationship between the potential of the postsynaptic membrane and the postsynaptic excitatory current. Furthermore, an increase in  $[Ca]_o$  produced an increase in the intensity of this current (800).

The definitive proof for the chemical character of synaptic transmitter in the squid giant synapse was obtained by Miledi and Slater (611). When  $Ca^{++}$  is removed from the environment transmission is blocked in spite of the unaltered invasion of the presynaptic ending by a normal action potential. In this condition

the only signs of electrical coupling between the pre- and postsynaptic regions are potential deflections smaller than 1 mV (611). In a low-Ca<sup>++</sup> medium normal synaptic transmission may be reestablished, as in the neuromuscular junction (464) if the arrival of the impulse to the nerve ending is synchronized with a microjet of Ca<sup>++</sup> released from a micropipette located near the synapse (611). In contrast, intracellular injections of Ca<sup>++</sup> into the nerve ending apparently have no effect on the transmission block, but this could be due to difficulties in increasing efficiently [Ca]<sub>i</sub> as a result of Ca<sup>++</sup> binding. Anyhow these experiments show the requirement of Ca<sup>++</sup> on the external surface of the ending membrane for normal synaptic function (464, 611). The effect of lowering Ca<sup>++</sup> is similar on both proximal and distal synapses (611). In both synapses increases of [Mg]<sub>o</sub> also blocks the transmission (611, 800).

Miniature EPSP's have been recorded from the squid stellate ganglion. Such a recording is not possible at the distal synapses of adult squids, probably because of the large size of the postsynaptic fiber (800). Miledi (607, 608) succeeded in recording miniature activity by impaling some of the neurons whose axons contribute to the formation of small stellar axons. The amplitudes of the mEPSP's vary from cell to cell and sometimes they are only just detectable above the noise level. Both their appearance at random intervals and their persistence after the abolition of the whole spike activity of the ganglion by TTX ( $2 \times 10^{-7}$ - $10^{-6}$  g./ml) demonstrate that they are not due to the firing of interneurons. It is also possible to record mEPSP's from proximal synapses (608). The miniature activity shows the same features there as in the distal synapses (94, 611). In young squid it has been even possible to record mEPSP's from the giant postsynaptic axons (608). The time course of the mEPSP recorded from the giant axons (rising phase: 1–2 msec) is different from the time course of the mEPSP recorded from the stellar neurons (rising phase: 5–30 msec). The causes for these differences are not yet clear (608).

3) *Mechanism of transmitter release.* The information summarized above demonstrates without doubt that the transmission at the giant synapses of the squid stellar ganglion must be chemical. The experiments changing [Ca]<sub>o</sub> and [Mg]<sub>o</sub> and the recording of miniature activity complete the similarity of the squid giant synapse with the vertebrate end plate. Therefore, the former offers an excellent preparation to test the Ca hypothesis of transmitter release, which has been postulated on the basis of converging evidence from work on cholinergic endings, adrenergic endings, and some endocrine mechanisms (see reviews in 195, 412, 460). According to this view during excitation Ca<sup>++</sup> moves across the ending membrane, following its electrochemical gradient or combined with a carrier, and then through some unknown reaction with the stores of transmitter Ca causes a transmitter release.

Experiments on synaptic transmission in the giant synapse in the absence of presynaptic nerve impulses (78, 79, 463, 464, 466, 467, 539, 540, 542, 543) have permitted further clarification of the mechanism of transmitter release. An important point established by these experiments is that presynaptic depolarization in the absence of a spike may still be able to evoke a postsynaptic potential change, provided that a certain amount of Ca<sup>++</sup> is present in the environment.

After the application of TTX ( $10^{-7}$ - $10^{-6}$  g/ml) to the giant synapse the presynaptic spike is abolished, but by applying short depolarizing pulses through intracellular microelectrodes the release of transmitter may be reestablished (78, 463, 464, 543) and the synaptic transfer characteristics (input/output) of such preparations may be studied. A postsynaptic response appears when the terminal is depolarized by 30 mv (463, 464) and the EPSP increases about 10 times with further 10-mv increments of presynaptic depolarization (463, 464). The efficacy of injecting large depolarizing pulses is limited by the phenomenon of delayed rectification (388) of the presynaptic membrane. Tetraethylammonium (TEA) ions intracellularly injected in the squid giant axon blocks the  $K^+$  channels and suppresses the delayed rectification (13, 351; see review in 382). TEA prolongs the presynaptic spike, increasing the release of transmitter (464, 542, 543). Large depolarizing potentials may be applied to the nerve endings under TTX and in the presence of TEA. In this condition presynaptic depolarizations longer than 10 msec evoke a maximal postsynaptic potential when they reach amplitudes of 75–100 mv (463, 464). Further increase in the amplitude of the presynaptic depolarization reduces the EPSP, which becomes completely suppressed by passing pulses of about 200 mv (463, 464, 543). This suppression-potential level, according to the calcium hypothesis, should correspond to the  $Ca^{++}$  equilibrium potential for the presynaptic membrane (463, 464). In the large depolarizations, in the falling phase of the pulse an "off" EPSP develops (464, 543). These off responses are maximal when the pulse reaches the suppression level. They may be interpreted according to the  $Ca^{++}$  hypothesis as follows. During the "on" part of the pulse,  $E_m$  is at the  $Ca^{++}$  equilibrium potential and no  $Ca^{++}$  enters into the ending. However, when the pulse is off the permeability to  $Ca^{++}$  is still high, and  $Ca^{++}$  enters down its electrochemical gradient (463, 464, 543).

As for the EPSP evoked by a normal presynaptic spike (93, 611, 800) only changes in  $[Ca]_o$  and/or  $[Mg]_o$  alter the synaptic transfer of the TEA- and TTX-treated giant synapses (464, 466, 467, 540). Neither changes in  $[Na]_o$  nor  $[K]_o$  affect the release (540). The synaptic transfer may be increased by previous short hyperpolarizations of the ending and depressed by predepolarizations (79, 464, 543). In the presence of TTX, high  $[Ca]_o$ , and after intra-axonal injections of TEA, a regenerative active electrical response of the axon terminals may be elicited and it may cause an additional release of transmitter (465). This TTX-resistant active response may occur in nerve endings placed in normal medium (465).

Strontium and barium may be substituted for  $Ca^{++}$  and the release is then partially maintained. The effect of  $Ba^{++}$  substitution is transient (466). Manganese ions counteract the action of  $Ca^{++}$  and this antagonism is probably competitive (466, 467). Lanthanum ions do not substitute for  $Ca^{++}$ ; on the contrary, they block the release of transmitter by presynaptic pulses even in the presence of  $Ca^{++}$  and abolish the tetrodotoxin-resistant regenerative response of presynaptic axons (583, 610). Lanthanum ions block the release without impairing the invasion of the endings by the action potentials (610). Under prolonged lanthanum treatment the endings show a progressive diminution of their synaptic vesicles (583).

The quantitative analysis of the relationship existing between the EPSP ampli-

tude and  $[Ca]_o$  shows that the transmitter release evoked either by a presynaptic spike or by pulses in TEA- and TTX-treated endings increases in a nonlinear fashion with the increase in  $[Ca]_o$  (467). By the mass-action law it is possible to demonstrate that this relationship is very steep and has a power function (3-4) similar to that found at the neuromuscular end plate (see 412). Lester (557) has also proposed a model in which the simultaneous binding of four  $Ca^{++}$  ions at the presynaptic ending would cause the release of a quantum of transmitter.

4) *Ionic mechanism of EPSP.* From early work by Hagiwara and Tasaki (351) it was known that activation of squid giant synapses was linked to a change in the postsynaptic membrane conductance. Some recent data give an idea about the possible ionic mechanisms involved, but it has not yet been possible to determine exactly the ions involved in this permeability change. This difficulty probably resides in the complicated geometry of the postsynaptic region, which makes data interpretation rather difficult. Moreover, the rectification properties of the postsynaptic membrane are an obstacle to the direct observation of an actual reversal of the EPSP.

A clear reflection of these complexities is the variety of measurements of the  $E_{EPSP}$  reported in the literature. In great part they were obtained by extrapolation of a straight line drawn from points relating  $E_m$  and EPSP amplitudes at values of  $E_m$  not reaching the delayed rectification level. The values thus reported range between +60 mv (351) and 0 mv (104, 540). Actual reversal values have been recorded only in occasional experiments (296, 609). Using voltage-clamp techniques Gage and Moore (296) measured postsynaptic current. In nine synapses, by plotting the values  $E_m$  (-160-+15 mv) against the intensities of the corresponding synaptic currents, a straight line could be drawn from the points thus obtained. By extrapolation of this straight line a reversal potential of the postsynaptic current of +45 mv may be calculated (296). Actual reversals of the synaptic current were occasionally seen at  $E_m$  values of +80 mv by Gage and Moore (296), who found a rather good agreement between the reversal potential calculated by them and the  $E_{Na}$  values of the squid giant axon, which is about +50 mv (383, 387, 620).

Miledi (609) measured  $E_{EPSP}$  values between +13 and +40 mv, observing actual reversals of the EPSP in three experiments. The current-clamp method was used and  $E_m$  could be varied in a large range because of the presence of TTX in the external bath, and the previous injection of TEA inside the postsynaptic fiber caused a disappearance of the postsynaptic spike and an attenuation of the delayed rectification phenomena (609). Transmitter release may be obtained then by presynaptic depolarizing pulses, and the points relating  $E_m$  to EPSP amplitude appeared in two different straight lines showing different slopes.

Extrapolation to a null value of the points obtained only by hyperpolarizing the fibers yields an  $E_{EPSP}$  value of -10 mv (609) but the actual reversals measured were always higher than +10 mv (see below for comparative data on the reversal potential of glutamate responses).

Gage and Moore (296) observed the effects of changing sodium around the synapse on the value of the reversal potential of the synaptic currents ( $E_{psc}$ ). In two experiments, when 50 % of  $[Na]_o$  was replaced by tris Cl, the  $E_{psc}$  shifted by

24 and 36 mv. These values are larger than the shifts expected from the Nernst equation (16 and 18 mv, respectively) (296). On the other hand, Kusano (540) observed very irregular effects of changes in  $[Na]$ , on  $E_{EPSP}$ . This author could measure a shift of 20 mv in only one experiment by reducing by half the  $[Na]$ . These irregularities may be due to geometrical complications of this recording or/and to difficulties in changing the solution around the giant synapses (see 557).

5) *Search for a transmitter.* Cholinergic agonists and antagonists have no effect on the SGS. It is necessary to apply very high concentrations ( $11^{-2}$  g/ml) of TEA, hexamethonium, or nicotine to cause impairment of synaptic transmission (93). The same is true for some amines like 5-HT, epinephrine, or tyramine (93). The only blocking agent effective in low concentrations ( $6 \times 10^{-5}$  g/ml) is procaine, which may cause a block of transmission associated with a slower rise of the EPSP without marked changes on the spike threshold (93).

However, a cholinergic system appears to exist in the stellate ganglion (629, 914). Acetylcholine, choline acetyltransferase, and AChE seem to be more concentrated in the region of the distal synapse than in the giant axon. Webb et al. (914) support the idea that the lack of effect of cholinergic agents may be due to the difficulty for the compounds to reach the active regions by passing across the thick connective tissue surrounding the synapse. However, after careful dissection or treatment with surface-active agents, ACh still blocks the transmission only at concentrations of  $5 \times 10^{-2}$  M in the presence of eserine  $10^{-4}$  M (914). For the time being these findings are not very consistent with a transmitter role for ACh at the giant synapse.

A) GLUTAMATE IONS. Glutamic acid is a very likely transmitter candidate in crustaceans and insects (see sect. ivB, vB). Because of its role in cell metabolism it is not surprising that it is one of the three more abundant amino acids of *Loligo* postsynaptic giant axons (180, 181, 507), but the biochemical composition of the presynaptic fiber is unknown. Glutamate ions applied by iontophoresis on the postsynaptic membrane of a giant synapse paralyzed with TTX depolarizes the membrane (608). The sensitivity of the synaptic membrane is not uniform, spots of high sensitivity being sharply localized, and the sensitivity fades when the pipette is moved across the surface of the axon (608). Intracellular injection of glutamate ions is ineffective in bringing any change to the synapse. Repetitive iontophoreric applications of glutamate cause a profound desensitization. These findings resemble those obtained in the neuromuscular junctions of arthropods (802, 882).

However, recent experiments by Miledi (609) would cast some doubts about a transmitter function of glutamate here. In the presence of TTX, to avoid postsynaptic spikes, and injection of TEA postsynaptically, to suppress delayed rectification, the reversal potentials of the glutamate responses differ from those values of the  $E_{EPSP}$  measured from the same preparations. For instance when the EPSP reverses at +28 mv the glutamate responses reverse around -22 mv (609). It is difficult to imagine that a difference in localization between the glutamate spots and the EPSP sites with respect to the recording micropipette could explain such different reversal values.

Some results more favorable to the glutamate hypothesis have been recently

obtained by Kelly and Gage (475). Glutamate concentrations of 5 mM do affect the rate of rise of the EPSP, and higher concentrations block the transmission. Since glutamate in these conditions does not seem to affect the membrane conductance of the postsynaptic axon, these results were interpreted as due to a desensitization of transmitter receptors by glutamate (476).

It is difficult to decide on the basis of this evidence whether glutamate may or may not be discarded as a transmitter candidate for the SGS; new evidence is necessary.

### C. Neuromuscular Junctions

Molluscan muscles have been classified in four groups according to their ultrastructure (362, 612): 1) cross-striated muscle, 2) regularly obliquely striated muscle, 3) irregularly obliquely striated muscle, and 4) smooth muscle. Our knowledge about the nervous control of these different types of muscle is very uneven, since there is much better information about the smooth muscles than about the other types (see 403).

*1) Structural background.* In the smooth muscles no band pattern is observed. Among these muscles the more interesting are the so-called catch muscles, which maintain the shells of lamelibranch molluscs closed for long periods of time. A typical example is the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* (see 941). The fibers of the molluscan catch muscles have a diameter 6  $\mu$  or more and are between 0.8 and 1.5 mm long (104). Teased fibers adhere in long, apparently unbroken, strands that may only be dissociated by teasing (104). The fibers are associated in bundles 40–200  $\mu$  in diameter, neighboring muscle fibers being linked by regions of tightly apposed membranes that appear as nexal junctions when the fibers are fixed in permanganate (104).

Two types of filaments were observed with the electron microscope in molluscan smooth muscles (567, 612, 853): thick filaments 10–30  $\mu$  long and 1600–1800 Å thick containing both myosin and paramyosin and thin filaments 80 Å in diameter containing actin.

After nerve stimulation or direct stimulation ABRM fibers contract; thereafter the muscle may stay contracted for long periods or may relax rapidly. The way the nerves control contraction or relaxation of ABRM is still not fully understood nor is the innervation of the ABRM fibers completely known. There appear to exist *a*) an excitatory system that initiates tonic excitation and *b*) a relaxing system that is able to suppress long-lasting tension (439, 568, 569, 850, 851). Excitor nerves enter in the midregion of the long muscle fibers and branch parallel to the muscle fibers (144, 851, 853). There is no precise information on how these nerves end, but since facilitation and summation of junctional potentials are observed (853) it is possible that nerve endings may be widely distributed. There have been claims (114, 797) for the possibility of stimulating the relaxing system independently from the excitor system and even for the existence of different axons mediating these actions (98, 797). The only firm ground in this sense would be the evidence suggesting the existence of cholinergic fibers innervating the ABRM that probably constitute the excitatory system (114, 377, 853, 855).

No neuronal bodies are associated with the ABRM (117). The nerve endings penetrate inside invaginations of the muscle membrane [Gori (cf. 856)]. They contain large granulated vesicles 700–800 Å in diameter (cf. 856). On the contrary, in the posterior adductor muscle of *Anodonta* only electron-lucent vesicles 400–1200 Å have been observed in the nerve endings (969). Around the endings gliointerstitial elements containing granules 2000 Å in diameter are observed. These elements probably belong to the gliointerstitial system (see 639).

In neuromuscular junctions of gastropods smooth muscles, presynaptic endings containing electron-lucent vesicles, granulated vesicles, and neurosecretory elementary granules have been described (45, 726, 727, 758).

2) *Contraction and catch.* ABRM bundles isolated from nervous ganglia respond to electrical stimulation with short pulses by brief twitches or by tetanus. The maximum tension developed in tetanus is 10–12 kg/cm<sup>2</sup>, which is about 3 times greater than in noncatch muscles (439). If the stimulation is done by passing long pulses or by applying acetylcholine (see below), tension is maintained for long periods after stimulation has ceased and resists stretch for periods as long as 30 min. This state of prolonged resistance to stretch in absence of any stimulation is known as "catch" (439, 441, 853, 854, 856, 860). As discussed above, there is evidence of a nerve input to the muscle whose stimulation could bring relaxation of catch. Since catch is observed after the "active" state has finished (439, 441) it is better to discard the concepts of "inhibition" or "inhibitory nerves" and to consider the existence of "relaxing" nerves.

Intracellular recording from ABRM (194, 375, 377, 855, 859) shows a resting potential of -65 mv, without rhythmical fluctuations. This resting potential would be mainly K<sup>+</sup> dependent; a straight line with a slope of 45 mv for a 10-fold change in external potassium is obtained when the resting potential is plotted against the logarithm of [K]<sub>o</sub> (855).

Submaximal stimulation of the byssus retractor nerve produces large depolarizations of amplitudes up to 20–25 mv, which may fall to half-value in about 500 msec. These depolarizations were considered excitatory junctional potentials by Twarog (855). These EJP's may show summation and facilitation and, when the depolarization reaches an amplitude above 35–40 mv, spike discharge and muscle contraction. The spike does not overshoot, only attaining the zero potential level (885). It may appear graded. In a Na<sup>+</sup>-free medium the ABRM fibers discharge a spike linearly dependent on the [Ca]<sub>v</sub> (859a). Tetrodotoxin does not affect the active responses of the ABRM fiber (855).

3) *Excitation and acetylcholine.* Acetylcholine was found to produce depolarization and contraction of the ABRM (114, 377, 850, 852, 855).

Acetylcholine is detectable in ABRM; Twarog (850) found approximately 1 µg/g tissue wet weight. Acetylcholinesterase could be detected with the Koelle method in large bundles of nerve fibers entering the ABRM, forming a longitudinal array along the nerve fibers and branching in Y-shaped patterns (114).

Recently Hidaka and Twarog (377) analyzed the effect of iontophoretic applications of ACh to ABRM. ACh ejected from a micropipette placed near the muscle fiber membrane and at a distance of about 100 µ from the recording micro-electrode evokes depolarizations that increase linearly in amplitude with the

increase of the iontophoretic current, finally reaching the spike firing level. When currents around  $5 \times 10^{-7}$  amp were passed during 50–100 msec the depolarizations decayed with a half-time of 10 sec (377). ACh responses may be evoked at every point of the muscle where the pipette approached, which shows that apparently the ACh receptors are located over the whole surface of the muscle fiber (377). These receptors show desensitization and may be readily blocked with  $5 \times 10^{-5}$  M dTC in around 10 min. Similar results were obtained with other solutions of ACh antagonists:  $10^{-5}$  M hexamethonium chloride;  $5 \times 10^{-5}$  M atropine sulfate;  $5 \times 10^{-5}$  M TEA bromide and  $10^{-7}$  M propantheline bromide (377, 855). Of the cholinesterase inhibitors, neostigmine potentiates the action of ACh whereas eserine blocks the receptors (377, 855).

The action of some of these blocking agents on the excitation of the ABRM is parallel to the action observed on the ACh receptors (114, 850). The actions of the ACh perfused onto the muscle in a concentration of 2  $\mu\text{g}/\text{ml}$  could also be blocked by hexamethonium, dTC, and atropine. Propantheline and methanteline also seemed to be the most active in this case (114, 855), but it cannot be overlooked that methanteline could have a presynaptic action in addition to blocking post-synaptic ACh receptors (855).

These effects support the idea that ACh possibly could be the transmitter of excitation to ABRM. This hypothesis is also supported by experiments showing a similar reversal potential of both the EJP and the ACh responses (377). Extrapolation of curves plotting the amplitudes of EJP and ACh responses against membrane potential gives reversal potentials of around -12 mv for the EJP and -13 mv for the ACh potentials. The participation of  $\text{Cl}^-$  in generation of ACh potentials may be ruled out (377). The removal of half the  $\text{Na}^+$  content of the saline and its replacement by tris Cl resulted in a diminution to  $\frac{1}{4}$  the ACh response, whereas a tripling of the  $\text{K}^+$  content caused a shift of the reversal potential of the ACh response to  $+12 \pm 7$  mv (377). These results indicate that ACh changes the membrane permeability to both  $\text{Na}^+$  and  $\text{K}^+$ , and the mechanism here appears to be similar to that observed at the vertebrate muscle end plate (799).

It therefore may be considered likely that ACh could be the transmitter mediating the excitation of ABRM.

4) *Muscle relaxation and monoamines.* It has long been known that catch tension in ABRM may be relaxed by nerve stimulation (797) or by repetitive square electrical shocks applied directly to the muscle (90, 257). A pure relaxation response of the muscle may be obtained when the excitation produced by direct electrical stimulation is blocked with ACh antagonists like propantheline. This indicates that the relaxation is mediated by nerves different from the cholinergic ones; 5-HT is present in ABRM in a concentration of 1  $\mu\text{g}/\text{g}$  tissue and perfusion of the muscle fibers with 5-HT at concentrations ranging between  $10^{-6}$  and  $10^{-9}$  M could provoke the relaxation of catch (850, 852). Twarog (850, 852) postulated that relaxing nerves acted on ABRM by releasing 5-HT, which would act not by inhibiting excitation but by specifically causing relaxation of catch (see also 98, 403, 853, 856). In the presence of 5-HT, tension lasts only the duration of the excitatory stimulus (408, 850).

5-HT specifically relaxes catch tension (850, 852, 856); the stretch resistance of the muscle falls to 50 % of the control value when it is perfused with a  $5 \times 10^{-8}$  M serotonin solution and to 20 % when 5-HT is perfused in a  $5 \times 10^{-7}$  M concentration. The stretch resistance is completely abolished by 5-HT at concentrations of  $10^{-6}$  M (856).

When the effects of 5-HT are examined in ABRM muscle fibers using intracellular recording 5-HT is observed to affect the threshold of the fibers, increasing their excitability (375, 856, 859a). The membrane potential of the fibers is unchanged in spite of the fact that effective membrane resistance decreases from a control value of 45–60 megohms (194, 859) to 23–45 megohms when the muscle fibers are soaked in  $10^{-7}$  M 5-HT (375). However, 5-HT does not appear to change the Cl<sup>-</sup>, Na<sup>+</sup>, or K<sup>+</sup> permeabilities (858). These findings confirm previous assumptions that 5-HT would cause an increase in the Ca<sup>++</sup> fluxes (613, 856); 5-HT was actually found to increase the rate of Ca<sup>++</sup> efflux from a resting muscle or a muscle under stimulation (613). In the resting muscle this efflux probably originates from a slowly exchanging compartment, probably corresponding to the intracellular compartment (169). It is also possible that intracellular free Ca<sup>++</sup> may decrease because 5-HT causes binding of this cation (613, 853, 854, 856), thus interfering with catch tension. There is independent evidence that 5-HT may diffuse into smooth muscle cells (85). According to this hypothesis the site of action of 5-HT would also be intracellular.

All these data are in favor of a transmitter role of 5-HT in the relaxation of catch in ABRM. It has been shown that 5-HT is present in molluscan neurons and their synaptic endings (see sect. IV A). Moreover, 5-HT has been detected in the pedal ganglion of *Mytilus* (921, 961) and at the ABRM itself (850, 961). Although amine oxidase has been described in the ABRM (77), no evidence of its physiological role has been obtained.

The receptors involved in the action of 5-HT on the ABRM differ from the molluscan neuronal receptors to 5-HT (see 315, 316). On muscle receptors LSD 25 has an agonist action, producing by itself catch relaxation (856). Bullard (98) has reported that bromolysergic acid (BOL 148) blocks the relaxing effects of direct nerve stimulation. However, BOL 148 does not seem to alter the effects of 5-HT on muscle tension (856).

Recently DA was found to show effects on the ABRM that resemble those of 5-HT: DA and 5-HT both increase the muscle excitability and relax catch (370, 376, 857). DA also hyperpolarizes the muscle fibers by increasing the membrane conductance to K<sup>+</sup> (370, 376). It has also been reported that DA would activate an electrogenic Na<sup>+</sup>/K<sup>+</sup> pump, since decreases in the outer Na<sup>+</sup> concentration and ouabain cause a disappearance of the DA hyperpolarization (376). These results must be taken very cautiously, since the blocking of the Na<sup>+</sup>/K<sup>+</sup> pump may cause a K<sup>+</sup> accumulation outside the fibers, thus changing E<sub>K</sub> and apparently producing a blockade of the DA hyperpolarization. In favor of such an interpretation is the finding that change in temperature does not affect the DA hyperpolarization (376).

The evidence summarized above suggests that 5-HT could be the substance

released by the relaxing nerves innervating the ABRM. More evidence is still necessary to accept such a hypothesis.

## VII. DISCUSSION

Having reached this point the courageous reader realizes that our knowledge of the transmitter role of a series of known compounds in the invertebrate nervous systems is still rather fragmentary and circumstantial. On the other hand, there are many indications that the "classical" transmitter molecules (ACh, NA, GABA, etc.) are not involved in many chemical synaptic processes. Some still undiscovered transmitter molecules must exist to mediate these.

Even though it may be too early to draw any conclusions about the evolutionary trends of chemical transmission mechanisms in invertebrates, it is tempting to speculate on some of these on a very provisional basis. Table 1 summarizes present knowledge on invertebrate neuromuscular transmitters. The reviewer provides there a high dose of optimism to accept the present candidates as the actual transmitter molecules. In fact, the evidence for transmitter function is circumstantial neuromuscular inhibition in all the phyla from nematodes to arthropods (all the phyla where peripherical inhibition has been confirmed). In molluscs no neuromuscular inhibition has been observed and only a relaxing innervation has been described in catch muscles. Relaxation in these muscles is not likely to be mediated by GABA and may be produced through a mechanism very different from postsynaptic inhibition (856). The evolution of neuromuscular excitation in invertebrates is much more complex. ACh may be the transmitter released by motor endings in lower invertebrates such as nematodes and annelids, but in arthropods the motoneurons are not cholinergic and a new molecule, probably glutamate, appears to mediate excitatory junctional transmission to muscles. Molluscs appear to follow the same trend found in worms, and ACh has been described in catch muscles as the likeliest candidate for excitatory transmitter (see sect. viC). ACh is also found in all vertebrate phyla as the transmitter released at neuromuscular junctions (460).

In vertebrates, inhibition has disappeared from the periphery and has been replaced by motoneuron inhibition at the spinal cord. Another interesting difference regarding the inhibitory mechanisms between vertebrates and invertebrates is

TABLE 2. *Transmitter candidates in invertebrate neuromuscular junctions*

	Nematodes	Annelids	Crustaceans	Insects	Molluscs
Excitation	ACh*	ACh*	Glutamate**	Glutamate*	ACh*
Inhibition	GABA*	GABA*	GABA***	GABA**	
Relaxation					5-HT ? DA ?

Number of asterisks (1-3) reflects the degree of reliability of available evidence on the transmitter function of the compound, 3 asterisks indicating the best evidence in favor of a transmitter role.

that in the invertebrate CNS (see sect. VI A) the same interneuron may mediate opposite actions (i.e. excitation or inhibition) on different postsynaptic neurons by releasing the same transmitter through different axon endings. Such a feature has not been found yet in vertebrate CNS, where in all the well-known cases synaptic inhibition is mediated by specific interneurons different from the excitatory ones (214). Moreover, no observations have yet been made in vertebrate CNS of an organizational feature found in certain invertebrate CNS, which consists of multiphasic synaptic actions that combine both excitation and inhibition elicited by the same transmitter, released from a single interneuron and acting on different receptors located on the same postsynaptic neuron.

At variance with these differences is a feature that seems common to both vertebrates and invertebrates—the high ionic selectivity of the postsynaptic membranes of chemical synapses. Table 3 summarizes the results on this aspect gathered in invertebrates. It appears clear that central and neuromuscular postsynaptic membranes of different invertebrate phyla show either an exclusive anionic or an exclusive cationic selectivity. The only exception appears to be the inhibitory synapses on the crustacean stretch receptor neuron, where it has been claimed that the transmitter, very probably GABA, changes the membrane permeability to both  $K^+$  and  $Cl^-$ . These results may be challenged on the basis that the effects attributed to changes in the  $K^+$  concentration in the environment can actually be due to changes in the internal  $Cl^-$  concentration (see sect. IV C). On the other hand, in view of recent results obtained in molluscan central neurons (sect. VI A), one would be more inclined now to interpret results showing that a transmitter increases the membrane permeability to both anions and cations as due to the activation of two different receptors, each linked to a different ionophore, instead of the activation of a single receptor linked to a nonselective ionic channel. It would be interesting

TABLE 3. *Ionic selectivity of postsynaptic membranes of known synapses*

	Site	Excitation	Inhibition
Nematodes	NMJ	Mainly $Na^+$	Mainly $Cl^-$
Annelids	CNS	?	Mainly $Cl^-$
	NMJ	$Na^+$ , $K^+$ , and $C^{+?}$	Exclusively $Cl^-$
Crustaceans	CNS	Exclusively $Na^+$	Exclusively $Cl^-$
	NMJ	Mainly $Na^+$ ( $Ca^{++?}$ , $K^{+?}$ )	Exclusively $Cl^-$
	SRO		$Cl^-$ and $K^+(?)$
Insects	CNS		
	NMJ	Mainly $Na^+$ ( $K^{+?}$ , $Ca^{++?}$ )	Exclusively $Cl^-$
Molluscs	CNS	Mainly $Na^+$	Exclusively $Cl^-$ or exclusively $K^+$
	SGS	Mainly $Na^+$ or $Na^+$ and $K^+$	
	NMJ	$Na^+$ and $K^+$	Relaxation $Ca^{++?}$

to reevaluate in this view the data obtained in vertebrate motoneurons, where synaptic inhibition is interpreted as due to an increase in the permeability to both  $\text{Cl}^-$  and  $\text{K}^+$  (214).

A long look at the evolution of the studies on chemical transmission in invertebrates, especially in the last two decades, leaves the impression that important progress has been made. The problems to solve are still enormous, but the trends to follow have become more and more clear and consist fundamentally of the anatomical, biochemical, and physiological identification of individual neurons and the physiological and pharmacological study of the connections between them and with the periphery. For these purposes methods of intracellular dye injection, microchemical assay, and microphysiological and micropharmacological analyses have been developed and many of their achievements have been shown in this survey. We can only hope that their use will solve the remaining problems in the near future successfully.

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