COMPARATIVE PHARMACOLOGY: NEUROTROPIC AND MYOTROPIC COMPOUNDS[1,2]

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This is the first time that the title *Comparative Pharmacology* appears within the covers of the *Annual Review of Pharmacology*. The *Annual Review of Physiology* has made it a practice, since 1951, to include each year a section on *Comparative Physiology*. It is well to pause and to examine the needs and meaning of the prefix "comparative." In the Introduction to his important text on comparative physiology, August Puetter (1) wrote in 1911,

> The knowledge of scientific laws, as sought by physiology, is reached by a process of abstraction of the common from the abundance of single phenomena. The activity which leads to the cognition of the common (general), is that of comparing and so every scientific work in the field of physiology and every work which transgresses the pure empirical establishing of single facts, is an effort in the sense of "comparative" Physiology. The comparing is the most essential method which we use in science. Does it, under these circumstances, make any sense at all to speak of "Comparative Physiology?" Are not . . . physiology and comparative physiology the same?

In the famous eleventh edition of the *Encyclopedia Britannica*, Ralph Stockman (2) investigates the scope of pharmacology in various countries and comes to the conclusion that,

> In English speaking countries and [with] the majority of German writers, [pharmacology is] the study of the action of chemical substances (as apart from foods) on all kinds of animals, from bacteria up to man; it is, in fact, a comparative study of the action of chemical bodies on invertebrate and vertebrate animals.

Add to this the more recent definition given by C. J. Kensler (3) in the *McGraw-Hill Encyclopedia of Science and Technology* (*1960*), . . . the science of detection and measurement of drugs and other chemicals on biological systems," and one may well ask, to paraphrase Puetter's question, "Does it make sense at all to speak of "Comparative Pharmacology"?

True meaning and intent of a term are not always the same. The term "comparative" has come to mean "nonmammalian" or "invertebrate", and it is with the understanding of this implication that this review has been

[1] The survey of literature pertaining to this review was concluded in October, 1964.

[2] The following abbreviations will be used: ACh (acetylcholine); ChE (cholinesterase activity); DCI (di-chloro-isopropyl-norepinephrine); DOPAmine (dihydroxyphenylethylamine); GABA (γ-aminobutyric acid); 5-HT (5-hydroxytryptamine); 6-HT (6-hydroxytryptamine); 5-H TP (5-hydroxytryptophan); and i.p.s.p. (inhibitory postsynaptic potential).

written. This does not mean that vertebrate data are ignored, but rather that the emphasis is on pharmacological observations and discoveries made on invertebrate organisms and preparations. The author wishes to stress that he believes in the true meaning of the term "comparative" and to express his hope that the time will soon come when findings made in studies on invertebrate organisms will be considered an integral part of pharmacology and that it will be found superfluous to single out comparative pharmacology as a special entity.

As indicated in the title, this article will be restricted to the action and interaction of chemical compounds (natural or synthetic) on nerve and muscle tissue.

Indole-alkylamines.—While the multiplicity of phenomena associated with 5-hydroxytryptamine (5-HT) in vertebrates has not been amenable to a simple definition of its role, there is a wealth of experimental data obtained with invertebrate animals that has suggested, to many, a definite role of 5-HT as transmitter substance. The first suggestion of this was the finding of J. H. Welsh (4) that 5-HT, in low concentrations, imitates the action of cardio-accelerator nerves on the lamellibranch heart. In the same year, Florey & Florey (5) presented the first evidence that 5-HT occurs in nerve tissue. The quantities found in molluscan ganglia, with the use of parallel bio-assay techniques (6), were later confirmed by Welsh (7) and Welsh & Moorhead (8), using chromatographic and fluorometric techniques. The latter authors gave extensive data on the quantities of 5-HT in nervous and non-nervous tissue of a great variety of invertebrates, and, most recently, Welsh (9) added interesting data on vertebrates.

Homogenates of molluscan nerve tissue (*Busycon*, *Venus*) were shown to have considerable 5-hydroxytryptophan-decarboxylase activity (10); it is, therefore, very likely that the 5-HT of molluscan nerve tissue arises through decarboxylation of 5-hydroxytryptophan (5-HTP).

Loveland (11) has now shown that iproniazid (isopropyl-isonicotinyl-hydrazide), a monoamine-oxidase inhibitor, enhances and that methysergide (1-methyl-D-lysergic acid butanolamide) blocks the action of the cardio-accelerator nerves to the heart of *Venus* (now called *Mercenaria*). Methysergide had previously been found to be the most effective antagonist of 5-HT, out of a large range of derivatives of lysergic acid (12). Loveland (11) attempted to detect an accelerator substance (5-HT) in the perfusate of hearts, the accelerator nerves of which were stimulated; the result was negative. This of course, does not disprove that 5-HT is the transmitter substance released by the accelerator nerves. Further evidence for this assumption is his finding that tachyphylaxis, induced by high doses of 5-HT (13), makes the heart insensitive to both 5-HT and accelerator nerve action. The specificity of 5-HT action on the *Venus* heart had already been thoroughly investigated by Greenberg (14), who studied the action of a large number of tryptamine analogues and suggested three negative binding sites for 5-HT.

On the whole, then, the evidence for 5-HT being a transmitter substance of lamellibranch cardio-accelerator neurons is perhaps as good as that for norepinephrine being the transmitter substance of mammalian postganglionic sympathetic neurons. But, as is the case with the catecholamines in vertebrates, the mechanism of action is still puzzling. There is, as yet, no evidence that these compounds exert their action exclusively on the postsynaptic membrane or that their effect can be accounted for by a characteristic permeability change towards one or the other ion. Investigations to detect such changes have not yet been made in molluscs. This is, in part, because of the fact that the action of 5-HT has been observed on cells of very small diameter which are not suitable for ordinary microelectrode techniques.

There is evidence, however, that important phases of 5-HT action take place within the postsynaptic cell. This is particularly true in the case of somatic muscle of molluscs.

The anterior byssus retractor muscles of *Mytilus edulis* are doubly innervated like other somatic muscles of lamellibranchs: one set of nerve fibers causes tension development, the other relaxation. Tension maintenance does not require activation of the muscle elements by motorneurons. This has been clearly shown by Twarog (15, 16, 17), who has demonstrated that ACh (acetylcholine) produces a contraction that is preceded and accompanied by transient depolarization; both are potentiated by anticholinesterases and blocked by cholinergic blocking agents such as tubocurarine, 3-diethylaminoethylexanthine-9-carboxylate methobromide (banthine), benzoquinonium chloride, and others. 5-HT, even at very low concentrations such as 10^{-9} molar, leads to immediate relaxation of tension. This relaxing effect is not accompanied by significant changes of membrane potential. If tension maintenance is not dependent on the level of membrane potential, 5-HT cannot achieve its effect by affecting membrane behaviour or by uncoupling; it must act directly on the tension maintenance mechanism [paramyosin (18)]. This is clearly an intracellular action. What it involves is, perhaps, suggested by experiments on another lamellibranch tissue: the gills.

Aiello (19, 20) and Gosselin (21) described the cilio-excitatory action of 5-HT on gills of *Mytilus*, *Modiolus*, and *Anodonta*. Aiello suspected that the cilio-excitatory substance present in gill tissue is 5-HT. He was able to confirm this by isolating 5-HT (about 0.5 μg per g) and 5-HTP (about 0.1 μg per g) from *Mytilus* gills by means of paper chromatography, spectrofluorometry, and bioassay (22).

In a subsequent investigation, Moore & Gosselin (23) found that 5-HT markedly stimulates anaerobic glycolysis, and Moore, Milton & Gosselin (24) reported that 5-HT stimulates endogenous respiration of isolated gills of *Mytilus* and *Modiolus*, quite in contrast to catecholamines and ACh which have no such action, even though the latter agent is known to accelerate the beating of the cilia [Bülbring, Burn & Shelley (25)]. 5-HTP was found to act like 5-HT, but the effect developed slowly; presumably the compound is first decarboxylated to 5-HT before the action becomes manifest. Most significant

is the finding that 2-bromo-lysergic acid diethylamide antagonizes the stimulatory action of 5-HT on ciliary respiration, while lysergic acid diethylamide (LSD) acts like 5-HT and is synergistic. This pattern of simulation of 5-HT by LSD and of block of 5-HT action by 2-bromo-LSD has been generally observed on various molluscan preparations: LSD, like 5-HT, stimulates the *Venus* heart (4, 7, 12, 14) and that of other molluscs (26, 27, 28), activates the rectum of *Venus* (29), the penis retractor muscle of *Strophocheilos* (30), and the noninnervated closer muscle of the glochidia larvae of the freshwater mussel, *Anodonta* (31), while 2-bromo-LSD antagonizes 5-HT in these preparations. The conclusion, first reached by Moore & Gosselin (23), that the excitatory actions of 5-HT on cilia can be explained by its effect on metabolism, appears very convincing. Could this also be the explanation of the effects on heart and somatic muscle? In order to answer this question, more must be known about the specific biochemical events responsible for the increase in anaerobic and aerobic metabolism.

An important step in this direction is the work of Mansour (32) on carbohydrate metabolism in the liver fluke, *Fasciola hepatica*. He found that 5-HT causes the formation of cyclic adenosine-3′,5′-phosphate from ATP. This compound activates phosphorylase, the rate-limiting enzyme of glycolysis [Sutherland & Rall (33) and others]. These findings of Mansour immediately call to mind the experiments of Axelsson, Bueding & Bülbring and others (34, 35) with catecholamines in smooth muscle. Here, the metabolic stimulation leads to increased sodium extrusion and, thus, to inhibition by membrane polarization. In the only molluscan preparation where membrane potentials have been measured [indirect technique, Twarog (17)], no hyperpolarization has been seen; in fact, 5-HT was found not to interfere with ACh or nerve-induced contraction: it only prevented maintenance of tension after termination of stimulation. Thus, a different molecular mechanism must be postulated, perhaps one that prevents crystallization of paramyosin.

Whatever the precise mechanism, it is important to realize that there is indeed strong evidence for an intracellular action of 5-HT. How does it get into the postsynaptic cell? This question has its origin in the conventional way of looking at synaptic transmission: a compound that has profound actions on effector cells is found in nerve cells, and nerve cells are found to have actions that duplicate those of the compound, so we assume the compound is a transmitter substance. However, the conclusion is not inescapable. What if the compound is present in both nerve cell and effector cell? It is quite conceivable that 5-HT is an internal regulator of cell activities and that a synaptic transmitter which is not 5-HT causes a momentary release of 5-HT from binding sites within the postsynaptic cell. Artificially applied, 5-HT would imitate the transmitter, not because it acts like the transmitter substance, but because it acts like the substance that is released within the postsynaptic cell in response to transmitter action. The question of how applied 5-HT gets into cells still remains, however, Experimental evidence indicates that it does so rather rapidly. The relaxing action on byssus retractor muscles of *Mytilus* is instantaneous.

Extracts of these muscles were shown (15) to contain about 1 μg of 5-HT per gram of tissue. If this amount were present in the nerve endings, the concentrations would be extraordinarily high. An investigation of the location of 5-HT should yield most valuable information and might shed light on the mode of action of endogenous 5-HT.

In connection with the possibility of 5-HT acting as an intracellular modulator of certain cell activities (metabolism, contractile events), the experiments on ciliary activity of lamellibranch gills are important. Of equal interest are studies on the rhythmically active closer muscle of the glochidia of *Anodonta* (31). This muscle is not innervated, yet applications of 5-HT or 5-HTP in low concentration cause increases in rhythmical contractions. Tryptamine is even more powerful, while tyramine, epinephrine, and norepinephrine cause very short lasting activation; that which is caused by tryptamine is prevented by iproniazid! LSD and chlorpromazine cause activation but prevent further activation by tryptamine. Lábos and his coworkers (31) believe that LSD and chlorpromazine compete with tryptamine for 'tryptamine receptors', and that tryptamine arises within the muscle cell in the course of metabolism.

Koshtoyants, Buznikov & Manukhin (36) believe that 5-HT is an endogenous regulator of ciliary activity in early embryos (veliger) of nudibranch molluscs. They find that 5-HT stimulates the beating of prototrochal and velar cilia, in concentrations as low as 10^{-16} g per ml. ACh, epinephrine, and norepinephrine have no such action. LSD inhibits motility, but this can be restored by 5-HT. These experiments belong to the rare ones in which an antagonism between LSD and 5-HT has been observed in molluscs, but it must be mentioned that the authors applied 2.5×10^{-5} g of the drug per ml, while they found the threshold for 5-HT action to be 10^{-10} g per ml. In view of the enormous potency of LSD in other molluscan preparations, this depression of ciliary activity by LSD may well be unspecific.

The potency of 5-HT and of LSD in many molluscan preparations is indeed startling. Manukhin & Buznikov (37) make use of the sensitivity of nudibranch embryos to 5-HT in a new bio-assay technique for this compound. They were able to plot calibration curves through the entire range of 5-HT concentrations from 10^{-6} to 10^{-16} g per ml. The effect of the drug was quantitated by determining the number of rotations of the embryos per minute.

It is generally considered that the similarity of the actions of 5-HT and LSD (or their antagonism, for that matter) is due to the similarity of their chemical structure so that both can act on the same 'receptor molecule.' That the action of LSD outlasts that of 5-HT can be explained when it is assumed that 5-HT is metabolized, while LSD is generally not, or if it is, it is metabolized very much more slowly. Wright, Moorhead & Welsch (12) discuss the enormous sensitivity of the *Venus* heart to applied LSD. At a concentration of $10^{-16} M$ this causes maximal and persistent stimulation. In a bath volume of 10 ml, there are only about 600,000 molecules of LSD; since a venus heart has about 100,000 cells, only six molecules per cell are required to bring about

maximal stimulation. The authors point out that even fewer molecules must be necessary, since another heart, if brought into the same solution in which a heart had previously been maximally excited by the LSD present, will show similar excitation. With these considerations in mind, it becomes very unlikely that 5-HT or LSD act on receptors in postsynaptic cell membranes and cause opening of pores, the mechanism generally suggested for the action of transmitter substances. Permeability changes, if brought about by opening or widening of membrane pores, must surely involve more than six pores per cell!

The effectiveness of such extremely low concentrations of 5-HT and LSD may be a special feature of nudibranch veligers and *Venus* hearts, but the fact remains that, given the proper circumstances, very few molecules are needed to establish the characteristic action of these compounds. There is, as yet, no reason to believe that where higher concentrations are needed, the mechanism of action of these compounds is different. The higher concentration would, then, simply provide a greater chance for a few molecules to get to the appropriate site of action.

From this point of view, it is very surprising that molluscan nerve and muscle tissue contain relatively large amounts of 5-HT; the brain of *Helix aspersa* was found (38) to contain up to 4 μg per g wet weight, while the mantle contained 1 μg per g, and the heart 3 μg per g. The byssus retractor muscles of *Mytilus* were found to contain up to 1 μg per g (15), and the ganglia of *Venus* contained no less than 40 μg per g wet weight (8). Kerkut & Cottrell (38) found that 5-HT diffused, from freshly dissected *Helix* brain, into the bathing saline medium to give concentrations up to 10^{-7} g per ml. Although they did not state the volume of saline involved, it is clear that nerve cells must have released rather large quantities of 5-HT. This release could be interpreted as an artifact, due to cell damage during the excision of the brain. It was found, however, that when the first release of 5-HT had subsided, electrical stimulation of the excised brain again brought about the release of 5-HT (identified by its accelerating action on the *Helix* heart and the fact that this is blocked by 2-bromo-LSD and methysergide). If the 5-HT of mantle, heart, and other muscles were present only in the nerve elements that must inevitably be present in the excised tissue, these remnants of nerve tissue would have to contain fantastic amounts of it. It seems more reasonable to assume that the compound is present within muscle fibers. But if this is so, how can one explain that the addition of a few more molecules should induce an action, while the endogenous 5-HT does not? For example: if a large *Venus* heart weighs 1 g and contains 1.65 μg of 5-HT in its 100,000 muscle cells, each cell must contain no less than 10^{-13} moles or 2.3×10^{10} molecules of 5-HT. Welsh (4) found the threshold of 5-HT action to be at $10^{-10} M$. With a bath volume of 10 ml, there are 10^{-12} moles of 5-HT available to the heart and 10^{-17} moles to each of its 100,000 cells. If all of the 5-HT molecules actually attach to or enter the heart cells, each cell acquires about 10^6 molecules of 5-HT, i.e. only 1/100 of 1 percent of the 5-HT it already has. It

is interesting that Krnjević & Mitchell (39) calculated the output of each motor terminal on a rat diaphragm to be about 10^{-17} moles of ACh per impulse or about 10^6 molecules. The correspondence with the situation on the *Venus* heart may be startling, but is misleading: in the case of the 5-HT action on the clam heart, the stimulatory action persists for many minutes; furthermore, we know that it takes only 6 molecules of LSD to reproduce and to surpass it. The figure of 10^6 molecules in the case of *Venus* heart muscle cells is certainly a high estimate, because it is unlikely that all the 5-HT molecules of the bath are actually "consumed" by the heart. If, as seems to be the case, 5-HT acts within the cells (in contrast to ACh, which seems to act on the membrane), we must assume that its molecules can readily traverse the cell membrane, or that they are transported across it. The concentration gradient across the membrane would oppose entry of 5-HT unless the intracellular 5-HT is present in a bound form. Welsh (40) has found evidence that 5-HT in *Venus* ganglia is present in granules; the same may be the case in heart and other muscle cells that contain 5-HT. It is obvious that the endogenous 5-HT is physiologically inert, i.e., more than 99.99 percent of it, according to the above calculation. It is quite conceivable that the action of LSD consists in splitting-off 5-HT molecules from intracellular binding sites, for which it is very likely to have a high affinity. If its union with each binding site is only temporary, it might move from one to the other and, temporarily or permanently, "liberate" 5-HT molecules. In this way, the overt action of LSD would really be that of 5-HT.

There is an urgent need for more quantitative studies on the amounts and condition of endogenous 5-HT and the numbers of 5-HT and other drug molecules which are required per cell to achieve the already-described effects. In particular, we should know the changes in endogenous 5-HT which result from the application of "tryptaminergic" compounds and nerve stimulation. Because they contain relatively large amounts of 5-HT and 5-HTP and because of their enormous sensitivity to tryptaminergic compounds, molluscan tissues are ideal objects for such investigations, which can be expected to shed considerable light on the molecular mechanism of action of these important compounds.

If one remains within the conventional concept of chemical synaptic transmission and restricts one's concern to the question whether nerve cells release 5-HT at their endings on effector organs as a transmitter substance, he will find that the following favourable evidence has been published: (*a*) 5-HT and 5-HTP are present in nerve cells (8, 38); (*b*) ganglia contain 5-HTP-decarboxylase (10, 38); (*c*) upon stimulation of ganglia, there is detectable release of a 5-HT-like compound (38); (*d*) the action of 5-HT applied to a mollusc heart resembles that of cardio-accelerator nerves (4, 14, 26, 28); (*e*) the action of both is potentiated by iproniazid and is blocked by 2-bromo-LSD and methysergide (11, 12); (*f*) after treatment with high concentrations of 5-HT, the *Venus* heart becomes unresponsive to 5-HT and to the action of cardio-accelerator nerves (11, 13); (*g*) after immersion for 20 hr in a solution of

reserpine (10^{-5} g per ml), nerve-heart preparations loose sensitivity to cardio-accelerator action, but remain sensitive to 5-HT (11) (according to Loveland (11), reserpine administered over a period of 2 wk to *Mercenaria* (*Venus*) reduces the 5-HT content of ganglia to about one-half); (*h*) 5-HT excites selected ganglion cells within ganglia (41, 42) that have been shown to release, upon stimulation, diffusible 5-HT; (*i*) molluscan kidneys (*Helix*) convert 5-HT to 5-hydroxy-indoleacetic acid (38); and (*j*) molluscan tissues contain amino-oxidase, the enzyme that destroys 5-HT (10, 11, 43, 44).

The arguments, above, have been presented by various authors, notably Welsh (7), Kerkut & Cottrell (38), and Loveland (11); of these, only (*a*), (*b*), (*i*), and (*j*) are acceptable, without reservation. The others cannot yet escape a critique of the experimental evidence. The release of a 5-HT-like compound from cerebral ganglia of *Helix*, as detected by Kerkut & Cottrell, may stem from cells damaged in the dissection since the procedure unavoidably involves cutting of nerve cell processes. Stimulation of the excised brain may, simply, have damaged, or otherwise unfavorably affected, further nerve cells. The authors gave no data concerning placement of electrodes, frequency, intensity, and duration of applied stimulation. The failure to detect released 5-HT in the perfusion fluid after stimulation of cardio-accelerator nerves should be mentioned again in this connection.

Even though 5-HT has been found to occur in ganglia, there is no evidence that it occurs in nerve cells and not in glia cells, and we do not know whether 5-HT is present in the cardio-accelerator fibers.

The action of cardio-accelerator fibers may involve a sequence of events, such as (*a*) release of transmitter; (*b*) reaction of transmitter with postsynaptic membrane or with certain structures within the postsynaptic cell (the latter reaction may be a secondary event, produced by ions entering through the activated membrane); (*c*) release of endogenous 5-HT from storage sites; and finally (*d*) action of the freed 5-HT. If that is the case, applied 5-HT would simply imitate step (*d*). The transmitter substance proper need not be identical with 5-HT.

Potentiation by iproniazid and block by 2-bromo-LSD and methysergide of nerve action and of the action of applied 5-HT may again reflect imitation by 5-HT of step (*d*) of the transmission process.

Tachyphylaxis may well be due to exhaustion of the agent with which 5-HT normally reacts, within the postsynaptic cell, to bring about its effect.

The reduction of 5-HT content of ganglia brought about by reserpine and the concomitant reduction in sensitivity of the heart to cardio-accelerator stimulation are not proof that 5-HT is released as a transmitter from tryptaminergic nerve endings; it is quite likely that reserpine also releases endogenous 5-HT from storage sites within cardiac muscle cells. Arriving nerve impulses could not then release the usual quantity of endogenous 5-HT. Thus, the heart would exhibit reduced sensitivity to cardio-accelerator stimulation; it would, of course, remain sensitive to applied 5-HT.

Excitation of certain neurons within the snail brain (41, 42) by applied

5-HT may be interpreted as resulting from an intracellular action of 5-HT; in fact, the presence of 5-HT in ganglia may well be indicative of an endogenous role of 5-HT in certain types of nerve cells.

The findings concerning 5-HT and related drugs in molluscs (and *Fasciola*) are very reminiscent of those concerning catecholamines and related drugs obtained with vertebrate material. Many of the drugs that act on noradrenoceptive effector organs do so by affecting the stores of catecholamine within the organ, causing release (or preventing release) of catecholamine from storage sites. In certain cases, this has been found to be the mode of action of the parasympathetic transmitter substance, ACh. There is still a question as to what these stores really are: nerve endings, chromaffin cells, or what else. The possibility that the catecholamines are present within the regular cells of the effector organs (e.g., heart muscle cells) must be considered.

It is interesting, in this connection, to mention observations made on crustacean heart, concerning the mode of action of ACh and related drugs. ACh definitely is not the transmitter substance of the cardio-accelerator fibers (45), yet the following evidence can be and in fact has been, quoted in favor of the assumption that it does function as a transmitter: (*a*) ACh is present in crustacean nerve cells; (*b*) ganglia and nerves contain cholinacetylase; (*c*) the action of ACh resembles that of stimulation of cardio-accelerator nerves; and (*d*) crustacean tissues, in particular nerve tissue, contain cholinesterase. Statements (*a*), (*b*) and (*d*), correspond to statements (*a*), (*b*), (*i*) and (*j*) of the arguments, given before, that support the assumption of 5-HT acting as a transmitter substance in molluscs, in particular on the molluscan heart. They are clearly invalid, however, because ACh does not occur in cardio-accelerator nerves, and drugs that potentiate or block the action of ACh applied to the heart do not modify the effect of the cardio-accelerator nerves (45). This example, applied to the function of 5-HT in molluscs, demonstrates that even those arguments that are based on unquestionable experimental evidence do not, by themselves, support the assumption of a transmitter action.

It is not the aim of this review to be unduly critical, but rather to emphasize the complexities of the problems under study. The complications discussed here arise out of a great amount of experimentation that has yielded information that cannot anymore be encompassed within the classical concept of chemical synaptic transmission but requires an extended interpretation. The efforts of the research workers, whose published reports were discussed in the preceding sections of this review, have brought us to a crossroad where we may have to decide on a new course in our general approach to the action of neurons on other cells and to the function of those compounds that have been defined as, or at least considered as possible, transmitter substances.

Over the past few years, several publications were concerned with effects of 5-HT and other compounds on activity of ganglia and muscles of molluscs.

They did not attempt to elucidate the mechanism of action but, nevertheless, presented interesting information. Koshtoyants & Rösza (46, 47) applied 5-HT, epinephrine, norepinephrine, tyramine, tryptamine, and chlorpromazine to different ganglia of the snail (*Hexil pomatia*) and recorded the electrical activity from the ganglion to which the drugs were applied and from others which had intact nervous connections with it. Although their technique does not preclude accidental entry of the drugs into the blood stream, their findings suggest activation of different pathways by different agents; e.g., 5-HT applied to the pedal ganglion stimulated electrical activity not only in the treated ganglion itself but also in the cerebral ganglion, while norepinephrine and epinephrine were found to diminish not only the activity of the pedal ganglion to which they were applied but also that of the cerebral ganglion. On the other hand, application of the latter two compounds to the subesophageal ganglion caused excitation there as well as in the pedal and cerebral ganglia.

Similar to these investigations are those of Salánki (48) and of Puppi, (49). Salánki applied drugs topically to the cerebral ganglia of the freshwater mussel (*Anodonta cygnea*) and recorded the contractions of the posterior adductor muscle. Unfortunately, his technique did not preclude entry of the drugs, given in rather massive doses of 0.1 mg per ml (with the exception of 5-HT that was applied at a level of 0.01 mg per ml), into the blood stream (open circulatory system). 5-HT, tryptamine, L-tryptophan, and 5-HTP cause long-lasting rhythmical contractions, but a fall in tone. Similar effects, but of shorter duration, were achieved with epinephrine and norpeinephrine. Reserpine and iproniazid (0.1 to 0.5 mg per ml) caused prolonged (10 hr and 3 days, respectively) rhythmical contractions, chlorpromazine (2 to 3 mg per ml) induced a contraction that lasted for several hours, while LSD (0.001 mg per ml) gave rise to long lasting rhythmical activity which was accompanied by general loss of tone. The LSD action was comparable to that of 5-HT, but was irreversible. Puppi (49) applied epinephrine and norepinephrine to the posterior adductor muscle of "*Lamellibranchiata*" (presumably a confusion of the name of the whole class with that of the species used—it is likely that the work was done on *Anodonta cygnea*). The catecholamines were found to cause loss of muscle tone (inhibition), acting directly on the muscle and indirectly by stimulation of neurons in the cerebral ganglion. The latter action only was blocked by dihydroergotoxin. The electrical activity of the ganglia was monitored with the aid of an amplifier of a frequency response of 0 to 75 cycles per sec; consequently, the records obtained are difficult to interpret. It is stated that higher concentrations of the catecholamines depress, while lower ones stimulate, electrical activity.

Meng (50) described excitatory actions of 5-HT on the heart of *Helix pomatia*. Hill (51) reported similar results with hearts of representatives of two suborders of prosobranch gastropods (*Busycon canalicalatum* and *Strombus gigas*). He also did experiments on the radula protractor muscle of *Busycon* and found that 5-HT, as well as tryptamine, added to a contracted

muscle (effect of applied ACh), causes rhythmic contractions ("beating") and gradual relaxation. Similar rhythmic contractions in response to the combined action of ACh and 5-HT were observed by Fänge & Mattison (52) on the radula muscle of another gastropod (*Buccinum undatum*), and by Twarog (17) on the anterior byssus retractor muscle of the lamellibranch, *Mytilus edulis*. Duncan (53) published the results of experiments on the rhythmically-active penis preparation of the snail, *Limnea stagnalis*. In contrast to epinephrine, which, like ACh, caused a quick contracture with superimposed rhythmic contractions, 5-HT caused relaxation, and, starting from a lowered 'baseline', enhanced contractions. 5-HTP did the same, but it took some time before the action developed; this was taken to indicate prior conversion of 5-HTP to 5-HT. LSD was found to suppress rhythmical contractions and to prevent the action of subsequently applied 5-HT. In view of the previously published effects of the combined action of ACh and 5-HT, which gives rise to rhythmical contractions, it would have been interesting to know whether the organ contained any intrinsic cholinergic neurons.

In contrast to the widespread efforts of various investigators to establish 5-HT as a transmitter substance, Kahr (54) attempted to prove its role as a regular hormone in cephalopod molluscs. Bacq & Chiretti (55) had previously shown that 5-HT is indeed released into the perfusate of posterior salivary glands of octopod cephalopods upon stimulation of the nerve supply of the gland. Kahr studied the behavior of chromatophores in the skin after subcutaneous injection of various drugs into the intact animal and after topical application to isolated pieces of skin. He disregarded the well known double innervation of the muscle fibers which, by their contraction, pull the chromatophore to which they are attached into a flat disc. He assumed that the chromatophore, itself, is capable of active contraction and expansion. Those who have experimented with octopus are familiar with the unilateral blanching that occurs suddenly upon stimulation of certain nerve centers, a phenomenon that cannot be explained by the release of a hormone. Kahr found that 5-HT caused contraction of the chromatophores, while ACh caused expansion. This is a most important observation, even though the explanation of the drug action is inadequate. Kahr finds that faradic stimulation (inductorium) of the skin causes chromatophore expansion, and that during 5-HT action, more voltage is needed to achieve the same effect. ACh was found to counteract the effect of 5-HT. Epinephrine, ACTH, and histamine were found to be without effect. 2-Bromo-LSD caused moderate expansion, but did not antagonize 5-HT. The concentrations used were low (10^{-9} to 10^{-11} mg per ml); higher concentrations were found to damage the cells. We have repeated some of these experiments in our laboratory (unpublished) and found that 5-HT blanches isolated pieces of skin but that stimulation of the nerve supply of the chromatophore muscles of a given region causes the same expansion of the chromatophores before and after application of 5-HT.

Cephalopod chromatophores provide a good opportunity for the assess-

ment of the mode of action of 5-HT. The compound is very likely to act on the chromatophore muscle fibers; the chromatophore, itself, is a convenient index for their overall length. The results to date permit the conclusion that the action of 5-HT in chromatophore muscles is similar to that observed with byssus retractor muscles of *Mytilus*. In both cases, 5-HT causes muscle relaxation but does not prevent ACh-induced muscle contraction. It should be possible to compare nerve-induced relaxation (blanching) with the action of 5-HT.

Concerning the role of 5-HT as a hormone released by salivary glands of cephalopods, it must be remembered that this compound does not occur in the salivary glands of *Octopus macropus*, or *O. defilipii*, or in those of *Sepia officinalis* and *Loligo vulgaris* (56). For this reason, it may not be necessary for the endocrine control of cephalopod chromatophores. Sereni (57, 58, 59), had suggested tyramine as an important endocrine product of *Octopus* salivary glands. He found it to have a stimulating action on nerve centers responsible for the darkening of the skin.

Mirolli & Welsh (60) have most recently published an account of their experiments on numerous species of molluscs, in which they studied the effects of injected LSD and reserpine on the behavior of the animals and on the 5-HT content of their ganglia. The behavioral changes obtained with reserpine (threshold amounts: 0.025 to 62.5 μg per animal) became evident 2 to 4 days after administration of the drug and consisted in general of immobility, tonic contraction of smooth muscle sheets of peripheral lacunae, and complete relaxation of the muscles of the shell. The peripheral hemostatic pressure was reduced, that of the visceral cavity increased. The effect was found to be temperature dependent: it occurred in *Melongena corona* after 3 days at 25° C, after 7 days at 16.5° C, and not at all at 9° C. Reserpine reduced the 5-HT content of the ganglia to nearly one third within the same periods of time, after which the behavioral symptoms appeared. No reduction of 5-HT took place at 9° C.

Animals treated with LSD showed general swelling, particularly in the periphery. The authors interpret the action of reserpine as being attributable to the release of 5-HT. It is not clear from their discussion, whether they favor the view that it is the actually released 5-HT or the lack of 5-HT that is responsible. They argue:

> If reserpine acts through the release of 5-HT and if, in particular, it is the continuous leakage of 5-HT across specific synapses that is effective in producing the syndrome shown by animals under treatment, then LSD cannot act at all the receptors involved by mimicking the amine, as it does in the molluscan heart. Rather one has to postulate that at some junctions the drug acts as an antagonist of 5-HT.

Clearly, the results quoted above do not bear out this hypothesis, since it would require the overt symptoms of reserpine administration to occur, while the 5-HT stores are being depleted, and not afterwards, as has actually been found. Lack of knowledge of the action of 5-HT, itself, makes an interpreta-

tion of the results difficult. The authors favor a role of 5-HT as synaptic transmitter. It must yet be established, however, that reserpine does not also deplete 5-HT stores in other tissues, such as muscle.

The occurrence and role of 5-HT in phyla other than the Mollusca, is very incompletely known. On the basis of bioassays, we had concluded (6) that the cardio-accelerator substance present in crustacean nerve tissue and in the pericardial organs is 5-HT. Welsh & Moorhead (8) did not find, by spectrofluorometric analysis, any 5-HT in nerve cord and peripheral nerves, but found up to 4 μg per g in the pericardial organs (neurosecretory structures) of *Cancer borealis* and up to 1 μg per g in the green glands (excretory organ) of *Cancer irroratus*. Maynard & Welsh (61) report to have found some 5-HT in ganglia of *Cancer borealis* (up to 0.08 μg per g) and up to 4 μg per g in pericardial organs, using spectrofluorimetric methods. Instead of 5-HT, Carlisle (62) found, in extracts of pericardial organs, a dihydroxy alkylamine which was later identified by Carlisle & Knowles (63) as 5,6-dihydroxytryptamine. In a reinvestigation of the problem of the identity of the indolealkylamine of crustacean pericardial organs, Kerkut & Price (64) found neither 5-HT nor 5,6-HT, but 6-hydroxytryptamine (6-HT). They found 6-HT ten times as active as 5-HT on the crab heart (acceleration). Identification was made with the specific color developed with Ehrlich's reagent, acid sulfanilic acid and alkaline sulfanilic acid, and in chromatography in five different solvent systems.

The effects of 5-HT on the heart of decapod crustacea are pronounced acceleration and increase in stroke volume [(6) and others]. Experiments in our laboratory (publication in preparation) have shown that the action is restricted to the ganglion cells of the heart and affects predominantly the dendritic endings within the heart muscle. 5-HT had been previously shown to excite receptor neurons of crayfish (65). 6-HT may well act in the same way, i.e., on nerve cells rather than on muscle. The presence of 5-HT in the excretory organs of crustacea (8) is very significant: (should this be now redefined as 6-HT?) It might indicate a role of this compound in water or mineral regulation. An antidiuretic action of 5-HT has been repeatedly described for mammals. Mirolli & Welsh (60) point out the breakdown of osmotic balance in molluscs treated with higher doses of LSD and reserpine and refer in this connection to the work of Mansour (32) and others [reviewed by Rall & Sutherland (66)] on the formation of cyclic adenosine-3′,5′-phosphate that is catalyzed by 5-HT (at least in *Fasciola*). Adenosine-3′,5′-phosphate has already been shown by Orloff & Handler (67) to imitate the action of antidiuretic hormone on the toad bladder. Earlier experiments of Erspamer (68) had failed to show any function of 5-HT and related compounds on water movements through the frog skin.

Welsh & Moorhead (8) had described the presence of relatively large amounts of 5-HT in abdomens or stinging segments and stinging apparatus of several insects, among them the honey bee. The values ranged up to 34.4 μg per g fresh weight (abdomen of a wasp, *Polistes*). Higher values (138 μg per

g) were found in the stinging segment of a scorpion, *Vejovis sp.* In the venom of the hornet, *Vespa crabro*, no less than 19 mg per g dry venom were found by Bhoola, Calle & Schachter (69). Welsh & Batty (70) have now added many more data on arthropod venoms and venom-containing parts. They found the venom of *Phoneutria* (*Ctenus*) *fera*, the most poisonous of the Brazilian spiders, to contain up to 2.6 mg per g dry weight. It is quite likely that the pain elicited by these venoms is due to the excitatory action of 5-HT on the terminals of sensory neurons, an action of 5-HT known since the studies of Armstrong and her co-workers (71).

5-HT was found by Boltt & Ewer (72) to be without effect on the lantern retractor muscle of a sea urchin, *Parechinus*.

The reader will recall that it was on a flatworm, *Fasciola hepatica*, that Mansour (32) found 5-HT and LSD to cause the formation of the phosphorylase-activating adenosine-3′,5′-phosphate. In the same animal, Mansour (73) had earlier found that 5-HT and LSD caused excitation and contractions; the effects were prevented by pretreatment with 2-bromo-LSD. Epinephrine and norepinephrine were without effect; they also were found to be inactive with respect to the formation of adenosine-3′,5′-phosphate. In another flatworm, the polyclad *Planocera*, Gruber & Ewer (74) found that 5-HT and LSD caused contraction of the longitudinal muscles of decapitated animals. No clear-cut results were obtained with 2-bromo-LSD, but ". . . the drug did appear to have no modifying effect on the responses to 5-HT or tryptamine."

The research literature on the occurrence and function of 5-HT in coelenterates has been reviewed earlier (75, 76). A recent statement by Welsh (77) that 5-HT ". . . is present in coelenteric tissues of *Caliactis parasitica* in very large amounts, but in other coelenterates it is most abundant in regions where nematocysts are concentrated," is somewhat misleading. The region 'most abundant in nematocysts' of *Metridium*, are the aconitia, filamentous appendages of the incomplete septa of the coelenteron. The whole coelenteric tissue of *Caliactis* was found to contain up to 600 μg of 5-HT per g dry weight, while the aconitia of *Metridium* contained 1.3 g per g (wet weight). According to Phillips & Abbott (78) 5-HT is not part of the nematocyst toxin.

Catecholamines.—DOPAmine (3-hydroxy-tyramine) has been found to occur in ganglia of various lamellibranch and gastropod molluscs [Sweeney (79)]. The amounts range between 26 and 261 μg per g, wet weight. Epinephrine and norepinephrine do not occur in molluscs (80, 81). Dahl et al. (82) have described an adrenergic nervous system in sea anemones. By a fluorescence technique, they observed adrenergic nerve cells in the tentacular apparatus of two actinians. Not only the cell bodies, but also their processes, and varicose terminals showed fluorescence after freezing and treatment with formaldehyde gas. The authors believe these to be sensory or sensory-motor neurons. The catecholamine is most likely identical with DOPAmine (Dahl, personal communication). It is interesting in this connection that Ross (83, 84) found DOPAmine entirely without effect on muscle preparations of sea

anemones, although he found epinephrine, but not norepinephrine, to cause tonic contraction and to increase responsiveness to electrical stimuli. Neither epinephrine nor norepinephrine has been detected in coelenterates (80).

Neurons containing catecholamines have also been found in the retina of rats (85) and rabbits. In the rabbit retina, the catecholamine has been identified as DOPAmine (86).

The actions of DOPAmine on crustacean stretch-receptor neurons and on spinal reflexes in mammals have given rise to much speculation. McGeer, McGeer & McLennan (87) found DOPAmine to be a most effective inhibitory agent that inhibited impulse generation in concentrations about a hundred times lower than the threshold concentration of γ-aminobutyric acid (GABA). While the action of GABA was blocked by picrotoxin, that of DOPAmine was blocked by chlorpromazine and dibenzylene. Since the authors found the latter blocking agents also effective against Factor I, McLennan speculated that the compound might, in fact, be the agent responsible for inhibitory actions of Factor I on the spinal cord. He did indeed find (88) that DOPAmine, topically applied to the exposed spinal cord of decerebrate cats, reversibly inhibited monosynaptic reflexes and that the effect could be blocked by strychnine and DCI (Di-chloro-isopropyl-norepinephrine). For a time, DOPAmine appeared to be an inhibitory transmitter. Curtis (89) has shown, by electrophoretic application to various types of spinal interneurons (including Renshaw cells) and motorneurons, that DOPAmine does not have an effect on the cells studied. He suggested that some of the DOPAmine that McLennan had topically applied to the cord may have been converted to the inhibitory substance during its passage through the tissues, or that, because of the similarity of its structure with that of the true inhibitory transmitter, the large concentrations applied by McLennan (2.5 to 5 percent w/v) could have operated the inhibitory synapses, while the smaller amounts, applied electrophoretically, may have been inadequate.

In a subsequent publication, McLennan (90) has provided evidence that DOPAmine excited certain interneurons in the intermediate nucleus of Cajal, while it was ineffective on any other interneurons tested. McLennan suggests that these are inhibitory neurons which cause inhibition of motorneurons. DOPAmine would, thus, inhibit reflexes by exciting inhibitory neurons. The excitation would be prevented by DCI; the action of the neurons would be blocked by strychnine.

McLennan & Hagen (91) compared the actions of DOPAmine on stretch-receptor neurons of four species of crayfish. They found it totally inactive on receptor neurons of *Procambarus blandingi* and of *Orconectes propinquus*, but 41.1 and 86.3 times as active as GABA on receptors of *Pacifastacus leniusculus* and *Procambarus clarkii*. These are very startling results, indeed. In our laboratory, we have never observed DOPAmine to be more active than GABA; at most, it had 50 percent of its inhibitory activity. The species used in our work was *Pacifastacus leniusculus*.

The normal role of DOPAmine in any nervous system (or effector organ, for that matter) cannot yet be assessed. It may well be a transmitter substance, but, as the examples of 5-HT and of other catecholamines (epinephrine and norepinephrine) show, the question of whether a compound is or is not a transmitter is almost naive, in view of the enormous complexity of their functions.

The inhibitory actions of DOPAmine on sensory neurons (crayfish) are most startling. If, like GABA, they are not blocked by picrotoxin, they must, presumably, act on different receptor structures. Does DOPAmine, in fact, act on the cell membrane and if so, does it affect the same permeability change as does GABA? Investigations to detect such permeability changes could shed considerable light on the mechanism of action of DOPAmine.

McGeer, McGeer & McLennan (87) also found L-epinephrine and L-norepinephrine to have inhibitor actions on stretch-receptor neurons. These compounds were found to be as effective as GABA. Elliott & Florey (92), working with a different species of crayfish, found these compounds to be without effect on stretch receptors. Using the same species of crayfish as was used by McGeer, McGeer & McLennan (87), we have never been able to observe any inhibitory actions of epinephrine or norepinephrine. The difference in sensitivity to these agents, therefore, may not be due to species differences, but to other differences in procedure, perhaps even in the history of the animals used. On the other hand, I have observed over the years that many stretch-receptor preparations are insensitive to 5-HT and ACh, while others, obtained from the same species, were strongly excited by these agents. Stretch-receptor neurons are surrounded by a dense sheath; it is possible that this interferes with the penetration of certain compounds, but it must be stressed that GABA never fails to exert its inhibitory action in concentrations that are always of the same order of magnitude.

While GABA appears to imitate the action of the natural transmitter substance of inhibitory neurons, which make synaptic contact with stretch-receptor neurons, catecholamines and ACh are unlikely to play a role in the synaptic excitation or inhibition of these nerve cells (93).

Numerous investigations have been concerned with a description of the overt actions of epinephrine and norepinephrine on numerous invertebrate preparations. They have recently been reviewed (75, 76, 94, 95). No serious attempts have yet been made to apply the extensive knowledge obtained with mammalian material to invertebrate preparations. It would be interesting, for instance, to have data concerning effects of the catecholamines on phosphorylase activation in those organs where they are effective; on mammalian heart the effects of catecholamines and sympathicomimetica on phosphorylase are parallel to those on the contractile behavior of the organ. At any rate, whoever works with catecholamines on invertebrate material should be prepared to conduct his investigations with a view of the importance of possible intracellular actions of these compounds and the agents that interfere with their action.

The role of acetylcholine and cholinesterase.—In Volume 2 of the *Annual Review of Pharmacology*, Crescitelli & Geissman (76) reviewed much of the literature concerning occurrence and role of ACh and the action of related drugs in the invertebrates. This section of the present review provides some supplementary information and discussion.

It is quite clear by now, that arthropod motorneurons are noncholinergic. Not only are the muscles insensitive to ACh, but motor fibers, at least in crustacea, have been found to contain no ACh (96). On the other hand, there is growing evidence that ACh acts as a central transmitter in arthropods, and that it is produced by sensory neurons.

In 1948, Roeder (97) discussed the possibility that ACh might be the transmitter substance released from endings of cercal nerve fibers in the cockroach. He found anticholinesterases to enhance, then depress, synaptic transmission to the giant nerve fibers in the ventral nerve cord; even high concentrations of ACh were without effect. Later, Twarog & Roeder (98, 99) were able to show that removal of the sheath from the ganglia made it possible to obtain effects with ACh. Even so, the effective concentrations were high ($10^{-2}M$ before, $10^{-3}M$ after, eserine), and the authors felt that ". . . the synaptic specificity of the acetylcholine action is still in question." More recently, Yamasaki & Narahashi (100), working with the same preparation, found that anticholinesterases (eserine, parathion, tetraethylpyrophosphate) enhance and prolong the excitatory postsynaptic potentials and increase the after-discharge. After variable intervals of time, synaptic transmission was depressed. ACh was found to stimulate postsynaptically the action being potentiated by anticholinesterases. Desheathing of the ganglion increased the sensitivity to ACh by a factor of ten. The authors came to the conclusion that ACh, or a similar ester, must be the transmitter substance of the cercal nerve fibers. It must be emphasized that these are sensory neurons. With crustacean peripheral nerve, it was found that its ACh is located exclusively in the sensory fibers (96).

In 1938, Bonnet & Bremer (101) described stimulation of electrical activity in the nerve cord of the crayfish by low concentrations of ACh administered by way of the blood supply of the central nervous system. Using topical applications of ACh, Prosser (102) found very little effect on spontaneous activity, even with high concentrations of ACh. Hichar (103) reports ACh (10^{-3} g per ml) to cause an increase in electrical activity of the fifth abdominal ganglion but does not state whether anticholinesterases sensitize the preparation towards ACh. Dimethylaminoethanol [shown to be a precursor of ACh (104)] in lower concentration (10^{-5} g per ml) caused irreversible excitation. Hichar (103) reports a startling correlation between ACh content and spontaneous electrical activity: he states that, "animals with 30 times more electrical activity also had 6000 times more ACh." Unfortunately, his presentation of results is difficult to interpret: He writes, "using normal activity in the last 100 experiments . . . the average number of impulses recorded from the fifth abdominal ganglion was 57/sec, with a minimum of

zero and a maximum of 447/sec." Then he says, "The variations in spontaneous activity of the 5th abdominal ganglion, was recorded from 10 animals . . . the difference between the average counts of the highest and lowest groups was 5300 action potentials/minute"—yet their levels of ACh were essentially the same—and, "Observations on 4 similar experiments gave comparable results." Only then does he report on "three other cords with little activity," in which he finds 0.001 to 0.0016 μg of ACh per g tissue. Further substantiation of these findings would be very important.

Lewis, Walker & Fowler (105) found more than twice the normal amount of ACh in the central nervous system of cockroaches, after prostration induced by excessive forced walking or by DDT. The cholinesterase activity (ChE) remained unchanged. The authors concluded that the increase in ACh content must be due to a release of ACh at abnormal sites; if released at normal sites, it would have been destroyed by ChE. The increase, could, of course, be brought about by increased synthesis and increased intracellular stores of ACh.

In a series of papers, Tauc & Gerschenfeld provided convincing evidence that ACh is a transmitter substance of central inhibitory neurons in *Aplysia* (106). The chief findings are: (*a*) presence of ACh and ChE in the ganglia (107, 108); (*b*) similarity of inhibitory postsynaptic potential (i.p.s.p.) and effect of applied ACh (same reversal potential when the membrane potential is artificially altered); (*c*) extreme sensitivity of nerve cells to ACh (threshold concentration 10^{-12} g per ml); and (*d*) block of i.p.s.p. and of ACh by *d*-tubocurarine.

Kerkut & Thomas (108) provided conclusive proof of the identity of the actions of the natural inhibitory transmitter acting on certain neurons in the abdominal ganglia of another gastropod, *Helix aspersa*. The reversal potentials of both i.p.s.p. and ACh were the same and changed in parallel when various ions were injected intracellularly or when the external potassium, or chloride ion, concentrations were altered. In view of the presence of ACh and ChE in the nervous system of this animal (38), it appears very likely that ACh is, in fact, the transmitter substance of inhibitory neurons. The specific inhibitory action consists in the permeability change to Cl^- (90 percent) and K^+. From the results of their numerous experiments, Tauc & Gerschenfeld (106) were led to the conclusion that the entire soma membrane, and not only the subsynaptic membrane patches of ACh-sensitive nerve cells (*Aplysia*), are cholinoceptive and that this entire membrane is both chemically excitable and electrically excitable. Tauc & Bruner (109, 110), described a desensitizing action of low concentrations of ACh towards later applications of ACh or actions of inhibitory neurons. Recovery may take (depending on the cell used) up to 30 min!

Experiments of Metcalf, Winton & Fukuto (111) on the cockroach heart indicated a high specificity of the receptors for ACh. ACh was found to be 40 times as active as acetylthiocholine or any of the other 11 related esters tested. Anticholinesterase agents, such as organophosphorous compounds

and phenyl-N-methylcarbamate, were found to accelerate the heart in great dilution, the action being prevented by atropine and pyridine-aldoxime-methiodide. The latter prevented the action of ACh. The authors assume a neurogenic pacemaker mechanism "incorporating synaptic transmission and having cholinergic properties."

It must be pointed out, however, that specificity to ACh does not necessarily imply cholinergic transmission. A good example for this is the heart ganglion of the crayfish, were ACh excites ganglion cells, and this effect is enhanced by eserine and blocked by atropine, yet there is no detectable ACh present in the cardio-accelerator fibers or in the ganglion cells, themselves. Atropine and eserine do not interfere with the action of cardio-accelerator nerves (112).

The action and normal function of ACh are not as simple as was once thought. It is true that there is excellent evidence for synaptic release of ACh on autonomic and neuromuscular synapses of vertebrates, and for a direct action on the cell membrane of vertebrate skeletal muscle and heart muscle. On heart muscle, however, the situation is not so simple; ACh was found to inhibit formation of adenosine-3′,5′-phosphate and of active phosphorylase, and the inhibitory action on the mechanical behavior was found to go parallel with this metabolic inhibition. One does indeed have to ask whether the intracellular action of ACh is a consequence of the membrane effects, or whether, in fact, the membrane action is caused indirectly by intracellular events.

In this connection, it is interesting to consider the findings of Köver & Kovács (113). These authors investigated, in tonic muscles, the relationship between myosin cholinesterase and ATPase activity, on the one hand, and ACh sensitivity, on the other. Myosin was prepared from skeletal muscle of rabbit and fish (*Ameiurus*), rectus abdominis muscle of frog (*Rana*), adductor muscle of mussel (*Anodonta*), foot muscle of snail (*Helix*), and longitudinal muscles of earthworm (*Lumbricus*) and leech (*Hirudo*). With the exception of the frog rectus abdominus muscle, it was found that the ACh sensitivity is directly proportional to the myosin-cholinesterase activity and inversely proportional to the ATPase activity of the particular kind of muscle. Muscles with high myosin-cholinesterase activity have low ATPase activity and vice versa. In muscles with low ATPase activity, ". . . ATP may play no direct, decisive role in the contraction". The authors suggest ". . . that in the native state, the contractile structures of the muscles possessing . . . a high myosin cholinesterase activity suffer changes in shape in response to acetylcholine, being acetylcholine receptor structures".

In the case of the rectus abdominis muscle of the frog, a high ATPase activity was found togehther with a low myosin-cholinesterase activity, although the muscle is very sensitive to ACh. Like other tonic muscles with high ATPase activity, this muscle contracts in response to applied ATP. This is not the case with muscles of low ATPase activity.

Szöör, Kövér & Pohánka (114) and Szöör, Kovér & Kovács (115) have

recently characterized rabbit myosin-cholinesterase with regard to substrate specificity and inhibition, investigating both the role of active anionic sites and that of the esteratic site. There are characteristic differences between true (ACh-)cholinesterase and myosin cholinesterase.

In 1953, Kirschner (116) described the effect of cholinesterase inhibitors and atropine on active Na^+ transport across the frog skin and suggested that ChE plays an important role in active transport.

In studies on the effects of anticholinesterases on the resting potential and on ion distribution in the sartorius muscle of frogs. Van der Kloot (117) was led to the conclusion that cholinesterase is intimately connected with active Na^+ transport across the muscle fiber membrane. Koblick (118), working on Na^+ transport through frog skin, came to similar conclusions and set up a theoretical ion-exchange model for active sodium transport, implying cholinesterase as the carrier enzyme.

Kövér et al. (119) demonstrated a positive inotropic action of purified cholinesterase on the hypodynamic frog heart. They provided evidence that oriented adsorption of cholinesterase "normalizes the state of the heart muscle membrane, the ion transport across it, and thus the contractile capacity of the heart muscle." Again, the experiments indicate a function in Na^+ transport.

Following the experiments of Koch (120), who had implicated ChE in the active Na^+ transport across isolated gills of the freshwater crab *Eriocheir*, Kamemoto (121) investigated the effect of eserine on Na^+ regulation in crayfish (*Orconectes virilis*). Injection of eserine into the animal was followed by a decline in serum Na^+ level, accompanied by an increase in urinary outflow of Na^+.

In a later paper with Keister & Spalding, Kamemoto (122) showed that the crayfish kidney contains cholinesterase and that the activity increased from the coelomo sac towards the bladder. The transfer of sodium from the lumen to the blood was inhibited, both *in vivo* and *in vitro*, after application of eserine. Thus, cholinesterase appears to be involved in the active reabsorption of Na^+ in the kidney tubules of the normal animal.

Fleming, Scheffel & Linton (123) found ChE in the gills of nine species of fresh or brackish water fish. They could not reach definite conclusions concerning the function of this enzyme. Eserine did not effect Na^+ uptake, but temporarily blocked Na^+ outflow.

Adding to this the well-known presence of ChE in mammalian erythrocytes (truly noninnervated cells), it becomes quite clear that the presence of cholinesterase is not necessarily associated with a cholinergic mechanism. One should, therefore, be careful in the interpretation of histochemical studies on the localization of ChE. Even if the enzyme appears to be true ChE or acetylChE, its presence cannot be taken as proof for the existence of a cholinergic synapse or for a role of ACh.

Gamma-aminobutyric acid (GABA).—The many papers concerned with studies of occurrence and function of GABA and some other amino acids,

notably glutamate and aspartate, have been reviewed so extensively in recent times (124, 125, 126, 127, 128, 129), that it remains only to add a few interpretations, comments, and statements.

Evidence for GABA being a transmitter substance in the mammalian central nervous system remains inconclusive if not negative, in spite of the application of refined techniques and formidable machines (130). The same can still be said about the role of GABA in crustacea in spite of the statement in a recent review that, ". . . the identification of GABA as an inhibitory transmitter in crustaceans, is now virtually complete" (131). Even the elaborate experiments of Kravitz, Kuffler, and co-workers (132, 133, 134) do not change this, although there can be no doubt that with appropriate techniques GABA can be obtained, in rather large amounts, from inhibitory neurons and, in smaller amounts, from excitatory ones. It has been stressed repeatedly that fresh extracts of nerve tissue, containing inhibitory neurons, contain a labile inhibitory agent. GABA, on the other hand, is very stable in extracts. Experiments in our laboratory indicate that the inhibitory agent of crab nerve tissue is an acidic compound (127, 135), the extractable labile activity being about 20 times more potent than could be accounted for by the GABA found by others (133, 134). Furthermore, we have now evidence [Florey & Iwasaki (136)] that the i.p.s.p. recorded from the soma of crayfish stretch-receptor neurons is normally hyperpolarizing, while GABA causes slight depolarization (KCl-electrode).

The difference between crustacean nerve tissue and that of mammals in which GABA can be readily detected (aqueous extracts) is underlined by the absence (135), or exceedingly small (137) activity, of glutamic acid decarboxylase in crustacean nerve tissue. The negative results of experiments in which thiosemicarbazide was injected into crayfish (135) may not be significant, however. Baltzer, Holtz & Palm (138) have shown that thiosemicarbazide does not lower the GABA content in mouse brains as do thiocarbohydrazide or isonicotinic acid hydrazide [in contrast to the earlier observations reported by Killam et al. (139)]. If the same were true in crayfish, this would explain the lack of effect of this drug and would remove the argument that GABA cannot play an inhibitory role in the crayfish nervous system. As has been explained elsewhere (136), our previous technique (135) of purifying the inhibitory agent(s) of crustacean nerve tissue was not suited to show the presence or absence of GABA in amounts less than 10 percent of that which was required to account for the inhibitory activity of nerve extracts. A new method for the purification of a rather active inhibitory substance (Substance I) from crustacean nerve has recently been described (136).

It is interesting that Kravitz et al. (133) found considerable quantities of GABA in crustacean muscle. An interpretation of their Figure 3, together with the information given in their method section, and their Table 1 indicates about 2000 μg of GABA per g dry-weight of lobster muscle, i.e., about 15 times as much as is found in peripheral nerve of the same animal. The relative volume of inhibitory nerve endings is very small compared to the

volume of the whole muscle; the ratio may well be larger than 1:100. It is much more likely that the GABA is present in the muscle fibers than exclusively in the nerve endings.

The problem of the transmitter substance of crustacean inhibitory neurons will not be solved until the nature and role of the unstable, but more potent, inhibitory factor of crustacean nerve tissue has been determined.

GABA was reported (140, 141) to change the membrane potential of crayfish stretch-receptor neurons and to have the same reversal potential as the natural transmitter (i.p.s.p.). This has been taken as evidence for its function as a synaptic transmitter substance.

In this connection, it is interesting that DelCastillo, DeMello & Morales (142) found GABA in low concentration to inhibit normally occurring pacemaker activity in nematode muscle and to increase specifically Cl^- permeability "like the natural inhibitory transmitter." The muscle fibers of nematodes are not innervated; rather, they send processes towards the central nervous system, where they form a "syncytium" that receives impulses from central neurons. The permeability change seen takes place at noninnervated membrane. There is no evidence for any neuromuscular inhibition in nematodes; certainly GABA exerts its action at a membrane free from synaptic contacts. Piperazine, an antihelminthic, acts like GABA, by increasing Cl^- permeability and, thus, paralyzing the worm (143). This example is quite pertinent to a discussion of GABA as an inhibitory transmitter in other animals, because it shows that a permeability change comparable to that produced (or expected to be produced) by an inhibitory transmitter does not even permit the conclusion that such a transmitter is normally acting on the membrane under study. Thus, the specific permeability change produced by a compound does not establish it as a transmitter.

In the light of this, the finding of depressant actions of GABA on electrical activity in ventral ganglia of caterpillars and annelids (144, 145, 146), or in cerebral ganglia of mussels (147), does not help much to ascribe a normal role to this compound in regulating, let alone synaptically inhibiting, the activity of ganglion cells of the organisms studied.

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