INVERTEBRATE PHARMACOLOGY: SELECTED TOPICS[1,2]

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We have been asked to review a subject with a vast background of unorganized and scattered literature which, to our knowledge, has never before been put together in the form of a unit. A number of books and summaries on specialized phases of this subject are available (1 to 11), and particular aspects have been treated in volumes on pharmacology (12 to 15), physiology or biology (16 to 24), comparative biochemistry (25 to 27), and miscellaneous publications (28 to 30). In the space alloted here we can touch only upon certain aspects which interest us. Topics such as invertebrate hormones, insecticides, invertebrate venoms, antibiotics, protozoan pharmacology, anthelminthics, the actions of drugs on nerve fibers, and others, have not been included here since these, in whole or in part, have been summarized in recent publications included in the list of the first 30 references. This review may be looked upon as initiating a series which periodically might make an accounting of matters in the field of comparative or invertebrate pharmacology. Our general aim is twofold: (*a*) to relate briefly, imperfectly known as they are, the pharmacological actions on invertebrates of some drugs of physiological interest and (*b*) to summarize the occurrence, pharmacology, and certain other relations of substances more or less unique to the invertebrate phyla. These two subjects have been treated separately for some substances but have been integrated wherever the demands of space and logic have required. In accord with current pharmacological trends, no attempt has been made to separate the strictly pharmacological elements of the subject from physiological, biochemical, and biological relationships. In fact, the attractiveness of invertebrate pharmacology for us is the interrelationship with these other disciplines. Though the major effort has been concentrated on the publications of the last five years, older fundamental reports have been included when required to develop a particular theme.

[1] The survey of the literature pertaining to this review was concluded in June 1961.

[2] Abbreviations used in this chapter include: ACh (acetylcholine); AChE (acetylcholine esterase); ChA (choline acetylase); GABA (γ-amino butyric acid); GSH (glutathione, reduced form); 5-HT (5-hydroxytryptamine); LSD-25 (*N,N*-diethyl-D-lysergamide); TMA (tetramethylammonium).

[3] Aided by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service, and by a grant from the University Board of Research. The authors wish to express appreciation to Miss Lou-Ann Roberts and to Miss Carole Kaufmann for their assistance in the writing of this review.

BIOASSAY METHODS

Occasionally, pharmacological knowledge has been advanced through the use of invertebrate preparations which have supplemented the usual vertebrate systems or offered special advantages in the detection and assay of pharmacological substances. The eserinized body-wall preparation of the medicinal leech introduced by Führer (31) and by Minz (32) is a simple, relatively sensitive, and, properly controlled and supplemented, fairly specific test preparation for acetylcholine (ACh) (33). In geographical locations where *Hirudo medicinalis* is not available, the possibility of employing other leeches has been suggested (34). The longitudinal body-wall muscles of sea cucumbers respond by contracture to added ACh, but not all species are suitable for assay purposes (35, 36). Muscles from some species show excessive spontaneous activity, lack specificity, or both. The uneserinized muscles of *Stichopus regalis* gave response to ACh at 10^{-7} to 10^{-8} and little sensitivity was found to histamine, epinephrine, or tyramine (35). A similar sensitivity was noted for the muscles from *H. nigra* (36) and *H. grisea* (37). The hearts of several invertebrates have been utilized in the assay of certain drugs. The heart of the lamellibranch, *Venus mercenaria* is very sensitive to ACh, responding to concentrations of 10^{-12} to 10^{-10} with a negative inotropic action (38, 39). This heart is relatively specific for ACh, showing less sensitivity to epinephrine, norepinephrine, tyramine, and histamine, (40), though indoles excite the heart and may interfere with the assay for ACh. Wait (41) called attention to the increase in sensitivity to ACh of the *Venus* heart as temperature was lowered from 30°C. to 10°C., a point of practical value in assays with this preparation. Several other molluscan species have been suggested for the assay of ACh (42, 43). In recent years the identification of ACh and other choline esters has been made more certain by the introduction of various electrophoretic methods (44, 45) and techniques of paper chromatography (46, 47). The *Venus* heart was also employed as a test object in the assay for 5-hydroxytryptamine (5-HT) to which the heart responds by a positive inotropic reaction (40, 48). Assay methods for 5-HT have been expanded by the introduction of sensitive and relatively specific techniques based on ultraviolet absorption as well as on activation and fluorescence spectra (49, 50, 51). These methods have been applied in the quantitative determination of 5-HT in the tissues of many invertebrates (52). The muscle stretch receptors of certain decapod crustaceans (53) have served as readily available test objects in the assay of γ-amino butryric acid (GABA) and related substances. The role which this receptor played in the factor I story is well known (54). Recently, Florey (55) employed the crayfish hind gut in the detection and assay of inhibitory substances like factor I or GABA. This preparation appears to be more sensitive than the stretch receptor but, like the latter, is not specific for GABA. The sea-urchin esophagus is not sensitive to GABA, but, like the stretch receptor, it does respond to factor I. This was one link in a chain of evidence which led to the conclusion that factor I is not GABA (56, 57).

The lantern of the firefly is known to be sensitive and specific to added ATP, and a method for the assay of ATP based on the light emitted (58) was recently employed to detect and measure the amount of ATP released at sensory nerve endings of the rabbit ear following antidromic stimulation (59). Finally, mention should be made of use of pig *Ascaris* in the testing of anthelminthics for human *Ascaris* (60) and the use of web patterns spun by spiders as a test for certain psychopharmacological substances (61, 62, 63).

PHARMACOLOGICAL ANALYSIS OF TRANSMITTER MECHANISMS

A large portion of the literature in invertebrate pharmacology deals with the effects of ACh, epinephrine, eserine, atropine, the curares, and pharmacologically related drugs. The major purpose of these studies has been, as in the case of the vertebrates, to elucidate the nature of the transmitter mechanisms in the invertebrates. In pursuing this aim, investigators have employed similar procedures and the same reasoning that went into the development of the neurohumoral theory for vertebrates. The outcome of all of this investigation has been that we have in a few cases good, though not conclusive evidence in favor of particular mechanisms of transmission; in most cases we know little or nothing about the mechanisms, and in no case do we have a picture as well developed as, for example, that of the vertebrate myoneural junction for the skeletal muscles. In a few cases through the use of invertebrate preparations, fundamental pharmacological information has been secured which is important to the student of vertebrate pharmacology—information which is difficult or impossible to obtain with vertebrates because of anatomical complexities.

Nonhumoral Transmission

Unpolarized junctions.—The giant axons of the earthworm and crayfish are provided with septa across which transmission appears to be bidirectional and explicable in terms of local circuit excitation (29).

Polarized junctions.—The giant motor synapse in the ventral nerve cord of the crayfish (*Astacus fluviatilis*) transmits excitation from the giant lateral presynaptic fiber to the smaller giant motor postsynaptic fiber. Transmission is unidirectional and apparently by means of local circuit, a conclusion that was checked by the use of internal electrodes placed across the synapse. Appreciable current transfer orthodromically occurred only when the subthreshold pulse in the prefiber was depolarizing. In contrast, antidromic current transfer was found only with hyperpolarizing pulses in the postsynaptic fiber. In addition, synaptic delay was short, about 0.1 msec. Transmission via a synaptic rectifier was postulated to occur (64). From a pharmacological point of view such a synapse might be expected to have properties different from those of a chemoreceptive synapse, and, indeed, Furshpan & Potter noted only insignificant effects of nicotine on this junction (64).

Cholinergic Mechanisms

There is an abundance of analytical data which suggests that a cholinergic system—meaning ACh and the associated enzymes for synthesizing and hydrolyzing this ester—is present in many invertebrates.

The occurrence of ACh.—Bacq (65) was one of the first to make a thorough comparative survey of the occurrence of ACh in invertebrates. Using the frog's rectus abdominis muscle according to the method of Chang & Gaddum (66), Bacq detected ACh-like activity in extracts of tissues from cephalopods, gastropods, lamellibranchs, polychaete worms, sipunculid worms, holothurians, and certain crustacea. The tissues of sponges, coelenterates, and ascidians gave little evidence of such activity. From the ganglia of *Octopus vulgaris* Bacq & Mazza (67) prepared the gold salt of ACh. Elementary analysis and melting point of the crystalline material agreed with the comparable data for the synthetic compound. This chemical identification, it will be recalled, was the first positive evidence that ACh does occur in nervous tissue, and it supplemented the earlier positive identification in horse spleen by Dale & Dudley (68). ACh-like activity was next demonstrated in terrestrial invertebrates (insects, arachnids) by Corteggiani & Serfaty (69). With leech dorsal body wall employed for assay, it was observed that high activity was present in the head region of insects and in nervous tissue. Identification of ACh in insects was made more certain by Lewis (70), who employed paper chromatography and identified in extracts of blowflies a material which moved inseparably with added ACh. The heads of about 23,000 honeybees were extracted, and the bases present in the extract were converted to the tetraphenylboron salts and to the reineckates (71). Separation by paper chromatography revealed the presence of substances in the positions of choline and ACh and of two additional unidentified spots. The substances in these spots behaved, chemically and biologically, like ACh. These were not acetyl-β-methylcholine, acetylthiocholine, or propionylcholine. The occurrence of ACh in insects was confirmed by Chefurka & Smallman (44, 72) who employed paper electrophoresis. Insect tissues have been found to have relatively high levels of ACh. Lewis & Smallman (73), for example, estimated that the brain of the fly (*Calliphora erythrocephala*) has 500μg/g of ACh, on the assumption that all of the ACh found in the head was in the brain.

Synthesis of ACh.—Extracts of tissues from several invertebrates have been shown to be able to make a substance with ACh-like activity (70, 74 to 78). The work of Smallman (77) provided evidence that the system in extracts of the blowfly head consists, as in vertebrate extracts, of both a coenzyme A-acetylating system and a choline acetylase. Lewis (70), using paper chromatography, and Frontali (45), employing electrophoresis and paper chromatography, showed that ACh was, in fact, formed by these extracts. Evidence of *in vivo* formation of ACh in houseflies was provided by Winteringham & Harrison (79) by the demonstration that the intra-

thoracic injection of 2-C^{14}-labelled acetate led to the appearance in fly extracts of labelled ACh, as indicated by paper chromatography.

Hydrolysis of ACh.—The presence of enzymes that split ACh has been detected in the tissues of many but not of all invertebrates (80, 81). Earlier work did not distinguish between the specific or true enzyme (AChE) and esterases of a less specific nature. Indications were obtained, however, that splitting of ACh was not a universal feature of all invertebrates. Bacq (82), for example, found no activity in coelenterates, in a sponge, in the blood and tissues of several crustaceans, or in the blood of insects and ascidians. More recent workers have definitely demonstrated the presence of the specific enzyme in invertebrates (83). Augustinnson (84, 85), for example, observed that the enzyme preparations of the squid cephalic ganglion, earthworm muscle, and *Helix* blood behaved in respect to ACh, propionylcholine, and butyrylcholine as did the AChE of vertebrate nerve tissue, muscle, and erythrocytes. An esterase was found in the trematode (*Schistosoma mansoni*) which split ACh three to four times faster than butyrylcholine, was inhibited by higher concentrations of ACh, but not of triacetin, and was inactivated by eserine or neostigmine (86). The concentration of the esterase was comparable to that found in mammalian brain. Centrifugation permitted Bueding (86) to separate the system hydrolyzing ACh from the system splitting butyrylcholine. Evidence of AChE activity was also found for the filarial worm, *Litomosoides carinii*, in muscle of *Ascaris* (86), and in the fresh water nemertean worm, *Prostoma rubrum* (87). Several workers have reported the presence of ACh-splitting enzymes, including AChE, in extracts of insects (88 to 92), in which activity in the head has often been found to be high (88). Certain invertebrates appear to have atypical esterases. The system from *Planaria dorotocephala*, for example, showed substrate specificities somewhat like AChE; yet, unlike AChE, it was not inhibited by higher concentrations of ACh, and it was much less sensitive than AChE to eserine (93). Finally, AChE was detected histochemically in the synaptic regions in octopus and squid and also along the fibers of the octopus stellate ganglion (94). The nonspecific cholinesterase was found in the connective tissue sheath of ganglia and nerves (94). Using the Koelle-Gomori method with acetylthiocholine as substrate, Wigglesworth (95) examined the distribution of AChE in the blood-sucking bug, *Rhodnius prolixus*. No evidence was secured for AChE at the end-plate regions of muscles, and, in contrast, eserine-inhibitable AChE was located in brain and ganglia, localized in the neuropile and between the axons rather than within the axons. No indications of pseudocholinesterase were found.

The cholinergic system in ontogeny.—The cholinergic system in invertebrates is not present in early embryonic development but appears after considerable tissue differentiation, especially of the nervous and neuromuscular elements, has taken place. No choline-acetylating activity was measurable for the first 24 hours of development, following oviposition, in

the Asiatic rice borer and the cabbage armyworm (96). Such activity, appearing first at the time of differentiation of the neuroblast, increased to a maximum until hatching. The first appearance of the choline-acetylating system coincided with the first indications of ACh-like activity. Cholinesterase activity was first observed 60 hours (in the rice borer) and 80 hours (in the armyworm) after oviposition. Mehrotra (97) made a careful examination of the sequence of development of ACh, AChE, and choline acetylase (ChA) in the house fly and the milkweed bug. Embryonic development is complete in 11 hours in the fly and five days in the milkweed bug. Acetylcholine was identified by paper chromatography, Hestrin's hydroxylamine-ferric chloride method, and the usual bioassay behavior. The nature of the esterase was established by substrate specificity tests. Neither ACh, AChE, nor ChA was found early in development in the fly or the bug. In the fly, ChA activity appeared five hours after ovipositon, AChE activity at seven hours, and ACh at nine hours. In the bug ChA was first noted at two days, whereas AChE and ACh first occurred at four days. Mehrotra concluded that the ChA developed at about the stage when the neuroblasts first formed. The eserine-inhibitable cholinesterase of the grasshopper (*Melanoplus differentialis*) was followed during three stages of the developing embryo: prediapause, diapause, and postdiapause (98). This system first appeared at about the seventh day of prediapause. It increased rapidly thereafter until the twenty-first day, at which level it remained throughout diapause and for the first five days of postdiapause; then it rose rapidly and continued to do so until hatching. The two periods of increase were correlated in time (*a*) with the origin and growth of the neuroblasts during prediapause and (*b*) with the secondary differentiation of these into a nervous system during postdiapause. No ACh-like activity was assayable during prediapause, diapause, or the first five days of postdiapause. On the seventeenth day of postdiapause ACh-like activity was noted. These results agree in general with the findings of similar investigations of the ACh-splitting enzyme system during development of vertebrates (99, 100). It is clear, however, that information is needed on the specific histological localization of the cholinergic system during embryonic development. Employing the Koelle-Gomori technique with acetythiocholine as substrate, Durante (101) attempted to get such information with the developing larva of the ascidian, *Ciona intestinalis*. No AChE action was noted for the early embryonic stages, but such activity appeared, coincident with the formation of the neurula, laterally and posteriorly in the two masses of presumptive muscle tissue. No other area of positive staining was noted, not even in the neural plate. The AChE response was retained exclusively in the region of the muscles throughout larval life until metamorphosis when the tail was resorbed leaving a positive response only at the posterior tip where a remnant of muscle tissue was retained. After metamorphosis no evidence of a positive reaction was obtained.

The invertebrate pharmacology of ACh and related drugs.—Invertebrate cells, tissues, and organs respond to ACh in several ways. In some systems

there is no obvious effect even at relatively large concentrations. These negative effects are often difficult to interpret since the presence of barriers or other complications, rather than the absence of ACh-sensitive cells, can always be invoked and this view is sometimes justified (102, 103). In other systems ACh, even in low concentrations, is an excitatory agent and the action is reversible. In still other systems, ACh acts as an inhibitory substance even in low concentrations, and once more the effects can be reversed. In a few cases a biphasic action has been reported, ACh exciting at very low concentrations and inhibiting at higher. The interpretation of ACh effects are often difficult to make and to justify. The target systems are seldom simple and usually not understood even morphologically. The responses of a cell may be direct ones to the drug or they may be indirect as in the case of innervated effectors. The existence of the innervating nerve cell may not even be suspected or its existence may be in dispute. These problems are not too dissimilar to those facing vertebrate pharmacologists.

One of the observations mentioned by Gaskell (104) in his memorable essay on the medicinal leech was that muscarine caused slowing, weakening, and even cessation of the pulsatile vascular vessels, effects which were antagonized by atropine. In other annelid worms (*Lumbricus*, *Arenicola*), it was noted that ACh stimulated the vascular vessels, an action which was potentiated by eserine and antagonized by atropine (105). The body-wall muscles of the leech are stimulated by ACh, especially after eserine (31, 32) and this effect is blocked by (+)-tubocurarine (33). True cholinesterase (AChE) is known to occur in leech body wall (106). The body-wall muscles of other annelids (*Lumbricus*, *Arenicola*) responded similarly to ACh, and eserine potentiated, whereas atropine diminished but did not abolish this response (107). A potentiation by eserine was demonstrated for the leech ventral body-wall responses to electrical stimulation of the ventral nerve chain (108). In addition, evidence was presented to suggest that nerve cord stimulation of the ventral body-wall preparation resulted in the appearance in the surrounding fluid of a substance preserved by eserine, which had ACh-like propeties on the leech dorsal body-wall preparation and on the cat blood pressure. Eserine also potentiated the responses to electrical stimulation in other annelids (*Arenicola*, *Lumbricus*) (108, 109). Though precise details are scanty, the results do suggest the presence of a cholinergic mechanism for the muscles of the body wall and possibly for the vascular vessels. There is one investigation, however, which emphasizes the tentativeness of the present situation. This is the study of Nicol (110) on the sabellid worm (*Branchiomma vesiculosum*). Nicol found that the body-wall muscles of this worm responded to ACh and that the contracture was potentiated by eserine but unaffected by atropine. Eserine, however, did not augment the contractures following stimulation of body-wall strips through the nerve cord or directly. Neither did (+)-tubocurarine block these responses.

In investigations of holothurian muscles, extracts of sea cucumber muscles have been reported to have ACh-like activity (65) and to be able to

split ACh (82) whereas the muscles themselves respond by contracture to small doses of ACh, an effect potentiated by eserine (35). This response was not specific, however, since tyramine, epinephrine, histamine, and adenosine also caused contractures potentiated by eserine (111). In fact, the response to K^+ was an eserine-augmented contracture (35). The response to direct electrical stimulation of the isolated muscle of *Stichopus regalis* was also increased by eserine and depressed by atropine. Finally, Bacq (112) reported the presence of a substance, behaving like ACh with the leech bioassay method which appeared in the bathing fluid after electrical stimulation of the *Stichopus* muscle. The nonspecific eserine effects are disturbing in these experiments, and one cannot insist that the results constitute unequivocal evidence for a cholinergic mechanism.

The anterior byssus retractor muscle (a nonstriated muscle) of *Mytilus* reacted to added ACh by a depolarizaton and a quick contraction which was followed by a slow relaxation (113, 114). Eserine potentiated these responses whereas atropine, benzoquinonium, hexamethonium, (+)-tubocurarine, propantheline (Pro-Banthine), and methantheline (Banthine) were able to block them. The last two compounds were especially effective in this antogonism. In contrast, atropine, hexamethonium, (+)-tubocurarine, and propantheline were ineffective in preventing the response to direct-current stimulation of the muscle. Histochemical examination showed the presence of cholinesterase-positive nerve fibers entering the muscle, but nerve cells or motor end plates were not seen (114). Twarog (113) obtained evidence for the presence of both ACh-activity and of AChE in extracts of *Mytilus* muscle. These data are in agreement with the hypothesis of a cholinergic mechanism acting by way of the nerve fibers, and the failure of the ACh antagonists to prevent contractions due to direct-current stimulation was explained (114) on the basis that such excitation was not via the nerve fibers but directly by the muscle fibers. Twarog (113) showed some interesting selective effects of epinephrine and 5-HT on the byssus muscle. Both these compounds, but especially 5-HT, were able to accelerate relaxation or to abolish the tonic phase of the response to ACh without reducing the initial rapid contraction. In fact the latter was augmented by these compounds. Extracts of byssus muscle gave no evidence of epinephrine-like activity, but a small amount of 5-HT was detected by bioassay. Twarog (113) expressed the idea that the byssus muscle has dual innervation, one set of fibers being excitatory and cholinergic, the other being inhibitory and mediating their action via 5-HT. The *Mytilus* muscle is worthy of further study if only for the fact that the action of 5-HT on this muscle is an unusual one, since this substance normally excites smooth muscles.

Perhaps the most illuminating experiments on cholinergic system are those of Tauc & Gerschenfeld (115 to 117) with certain nerve cells in the abdominal ganglia of the gastropod *Aplysia depilans*. These experiments illustrate the value of employing simpler cellular systems in pharmacology since direct and indirect effects may then be more easily recognized and the effects of drugs more intelligently evaluated. One result of this work was the

clear finding of significantly different pharmacological characteristics on the part of nerve cells, even contiguous ones. This discovery was made through the use of the technique of localized microelectrophoretic application of drugs to the cell membrane outer surface while recording internally with microelectrodes. Two types of cells were identified: H-cells provided with an inhibitory input and D-cells with an excitatory input. The inhibitory synapse of the H-cells was considered to be cholinergic because (*a*) ACh in low concentrations reduced or abolished the spontaneous discharge, (*b*) ACh mimicked the membrane changes produced by stimulation of the inhibitory axon, (*c*) ACh either depolarized or hyperpolarized depending on the level of the membrane potential, artificially varied by means of applied current, (*d*) (+)-tubocurarine and atropine antagonized the inhibitory postsynaptic potentials and the action of ACh, (*e*) eserine prolonged the inhibitory nerve effects and augmented the action of ACh, and (*f*) though ACh had similar effects on the membrane of the axonal synaptic field and on somatic, nonsynaptic membrane, the latter portion of the H-cell was much less sensitive to the drug. In conjunction with the finding of ACh-like activity and ACh-splitting activity on the part of the *Aplysia* nervous system (118), this array of evidence suggests that either ACh or a very similar choline ester is the transmitter of the inhibitory synapses in *Aplysia* ganglia. In contrast, the responses of the D-cells to ACh were very different. Acetylcholine depolarized the membrane and increased the rate of spontaneous discharge, whereas the sensitivity to ACh, though still high, was somewhat less for the D-cells. Eserine increased and (+)-tubocurarine blocked this excitatory action of ACh. The D- and H-cells were pharmacologically different in still other respects. Norepinephrine hyperpolarized and inhibited the D-cells while exciting the H-cells. Epinephrine acted similarly but was about one-fifth as active. 5-Hydroxytryptamine excited both, but the D-neurones were more sensitive to this substance. γ-Aminobutyric acid (GABA) usually excited and depolarized the H-cells while inhibiting and hyperpolarizing the D-neurones. This action of GABA is especially significant since it illustrates an inhibitory action by this amino acid on neurones which apparently have no inhibitory input. The pharmacological differentiation of these two types of nerve cells is indeed revealing, though the physiological significance, if any, is still uncertain. This elegant study highlighted some of the difficulties involved in the interpretation of results obtained by mass administration of a drug into a population of neurones with different pharmacological properties. Generally an excitatory agent when administered locally, 5-HT was found to produce inhibiton of H-cell discharge when perfused through the whole ganglion, presumbaly by exciting an inhibitory interneurone to the H-cell.

The nerve cells in the supra- and subesophageal ganglia of the marine snail, *Onchidium verruculatum*, are provided with both excitatory and inhibitory inputs. Stimulation of the inhibitory prefibers caused typical postsynaptic membrane changes (119). γ-Amino butyric acid, γ-amino-β-hydroxybutyric acid, and β-alanine were without effect on these cells. This

is unlike the action of theses substances on crustacean stretch receptors and crustacean myoneural junctions, to be described later. Only ACh and γ-aminobutyrylcholine produced inhibitory action similar to the inhibitory neuronal effects. We have here an example of a nerve cell provided with inhibitory junctions which is inert to GABA.

A very large portion of the literature in invertebrate pharmacology is concerned with the heart. This subject has been reviewed on a number of occasions (120 to 123), and we shall be concerned here with only a general summary of the subject along with some recent findings.

The hearts of many arthropods are known to be accelerated in response to added ACh (124). This action has often been shown to be potentiated by eserine (125 to 128) and to be blocked by atropine (127, 128 to 131). The effects of curare are uncertain, some authors claiming no antagonism by the curare substances (128), whereas others reported that curare previously applied to the heart prevented or modified the action of ACh (121, 127). All these results have been interpreted by some investigators (121) to indicate a role for ACh in the control of the arthropod heart beat. Prosser (124), for example, conceived the excitatory action of ACh to be an effect on nerve elements which originate the rhythmic beat. In support of this view Prosser cited the lack of effect of ACh (even after eserine) on the noninnervated embryonic *Limulus* heart, whereas the adult neurogenic heart responded to ACh with a typical acceleration. Prosser's concept was tested in an elegant study by Garrey (126) who employed the adult *Limulus* heart in which the circumstances of anatomy permitted the independent pharmacological testing of the superficially located cardiac ganglion and the anterior two cardiac segments which are separated from the ganglion though innervated by it and driven by its rhythmic discharge. It was found that ACh applied to the cardiac segments, even after eserine, was ineffective in modifying the beat. Acetyl-β-methyl-choline and carbaminoylcholine were likewise without effect. The myoneural endings of the *Limulus* heart are probably not cholinergic, and Krijgsman (121) considered these motor nerve endings to be adrenergic. In contrast, ACh applied directly to the cardiac ganglion, especially after eserine, caused a definite excitatory action as recorded by the beat of the untreated anterior cardiac segments. No evidence of atropine antagonism to ACh was secured. Carbaminoylcholine was especially effective in stimulating the cardiac ganglion, although eserine was unable to enhance its action. Although admitting the striking effects of ACh on the ganglion, Garrey refrained from interpreting these as evidence of the existence of a cholinergic mechanism in the normal functions of this neurogenic system. Exceptional cases have been noted in which ACh produced inhibitory responses in the heart (132, 133). The *Daphnia* heart was considered to behave more like the myogenic heart of vertebrates and molluscs than the neurogenic arthropod heart (133). It should be noted, however, that in this experiment the whole animal was immersed in the test solution and the effects on the heart were assayed by observation. The possibility of indirect actions and other complicating effects makes this result difficult to compare

with experiments in which the heart was either bathed in, or perfused with ACh solutions.

The molluscan heart beat is generally considered to be myogenic in origin and to resemble the vertebrate heart rather than the arthropod heart (123). Acetylcholine in small concentrations produces reversible inotropic and chronotropic depression, and this action does not appear to be on the contractile mechanism (38, 123). Atypically, the hearts of molluscs of the genus *Mytilus* are excited by ACh (134). The threshold range of concentration over which inhibition occurs varies considerably from species to species, the variation being as much as 5 to 6 log units. Eserine has a variable influence on the ACh action, augmenting it in some species (38, 135 to 137) and having little or no influence in others (36, 42, 136, 138). These differences may be the result of varying amounts of cholinesterase in molluscan hearts. Smith & Glick (139) noted the low ACh-splitting activity of extracts of the *Venus* heart, which was reported to show little increase of the ACh action by eserine. There is general agreement on the point that atropine, though having actions of its own, is unable to antagonize the inhibitory effects of ACh (38, 42, 122, 123, 135). Heymans (140) noted this absence of antagonism for the heart of the gastropod, *Aplysia*, a point which was confirmed by von Euler, Chaves & Teodosio (141). Similar negative results were secured by Bacq (142) with the heart of the squid, *Loligo pealii*, and by Jullien (143) who employed the snail, *Murex*, and the oyster. While admitting the existence of nerve cells in *Murex*, Jullien (143) decided that the negative effects of atropine made it impossible for him to apply to the molluscan heart the cholinergic mechanism of the vertebrate heart. Curare also has effects of its own on the heart of molluscs, but there are many indications that it is not a very effective antagonist for ACh (42, 123, 144, 145). On the other hand, mytolon, 2,5-bis-(3-benzyldiethylammoniumpropylamino)-*p*-benzoquinone chloride, was discovered to be a very active ACh antagonist for the hearts of certain molluscs (42, 144, 145, 146). In respect to ACh-blocking agents, the molluscan heart appears to be pharmacologically different from both the myoneural junction and the autonomic ganglia of vertebrates (144, 145). Occasionally ACh has been noted to have a biphasic action, exciting the heart at low concentrations and causing inhibition at higher concentrations. Such a result was published by Corda (147) who observed a definite amplitude increase in the beat of the uneserinized heart of *Helix aspersa* at lower concentrations of acetylcholine bromide. The notable feature of this result was that ACh at 10^{-17} g/liter was shown to be active in exciting the heart. Corda was of the opinion that ACh in small amounts is a substance of physiological significance in the beat of the *Helix* heart. This is not the view of Jullien, Ripplinger & Cardot (148) who preferred to view ACh as a toxic by-product without physiological influence. Corda (147) pointed out, however, that Jullien's group had not employed ACh at concentrations lower than 10^{-8} which is necessary in order to obtain, according to Corda, the excitatory actions of ACh.

Fredericq (122) reviewed the status of the mediator hypothesis as applied

to the mechanism of action of the cardioregulator nerves of invertebrates. The subject was shown to be in a very tentative state. Little progress has been made since then and no one has yet identified a chemical substance specifically associated with the process of mediating the action of the regulator nerves, although indications of a cholinergic mechanism have occasionally been published. Prosser (38) collected the eserinized sea water which bathed the heart of *Venus* during inhibitory stimulation and tested it on an eserinized recipient heart. A definite amplitude reduction was produced in the beat of the latter heart. On the basis of this Prosser argued in favor of a cholinergic mediator for the process of inhibition. This suggestion was supported by the observations that eserine intensified both the action of added ACh and of nerve stimulation, whereas atropine was unable to block either the effects of inhibitory nerve stimulation or of added ACh. Welsh supported the Prosser experiment by showing that the inhibitory effects of direct electrical stimulation were intensified by means of eserine and diminished by use of an ACh antagonist, tetraethylammonium (134). With the clam (*Anodonta*), Ten Cate (149) noted that inhibition brought about by electrical stimulation of the heart was abolished by atropine. Prosser's results still appear to be the only direct demonstration of the liberation during nerve stimulation of a substance mimicking the effects of cardiac nerve excitation. They have neither been confirmed in *Venus* by the identification of a definite substance, nor have they been extended to other species. With regard to arthropods there is the recent statement by Florey (24) that ACh is absent in the accelerator nerve fibers and cardiac ganglion of the American lobster and that atropine and eserine have divergent influences on the actions of ACh and of accelerator nerve stimulation on the lobster heart. This casts doubt on the role of ACh in neural control of the heart of decapod crustacea.

Acetylcholine: Other Functions

As in the case of vertebrates where ACh is known to occur in such unusual locations as the placenta (150), so also in certain invertebrates ACh has been detected in structures involving no obvious nerve activity. Traces of this substance have been reported to occur, for example, in the hypobranchial gland of certain molluscs (151) and in the posterior salivary glands of octopods (152). Hornet venom has also been reported to have ACh-like activity (4). Perhaps the most intriguing of these locations is the royal jelly of the honeybee where ACh was detected in relatively large quantities (800 μg/g) (153) Paper chromatography of extracts of royal jelly revealed the presence of two spots identifiable as choline esters. One of these was identified as ACh. The second was in the position of butyrylcholine, but differential bioassay methods showed it to be pharmacologically unlike butyrylcholine. Preparation and chromatographic separation of the reineckates, high-voltage electrophoretic separation of the choline esters, and identification of the acid component of the esters revealed only one choline ester, ACh, present in the extract. A nonspecific cholinesterase was also detected associated with an

enzyme fraction separated by electrophoresis (154).[4] The role of such significantly large quantities of ACh in such a biologically important material as royal jelly is intriguing but unknown. The composition of royal jelly is complex and only partly understood (154, 155). The difficulties in obtaining reliable data for the composition of royal jelly are, first, the problem of securing samples uncontaminated with honey or other materials and, second, royal jelly is unstable and will change on standing. The composition with respect to amino acids and sugars has been reported (154, 155). Royal jelly is deficient in certain vitamins and high in others, especially certain B vitamins. The content of pantothenic acid is unusually high, exceeding values for yeast or liver (156). Royal jelly also contains a unique pteridine [2-amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)-pteridine] (30) and an unsaturated fatty acid (10-hydroxy-2-decenoic acid) (157, 158). The nature of the substance or substances in royal jelly responsible for differentiation of a bee larva into a queen, assuming this to be the mechanism, remains undiscovered. Callow & Johnston (159) concluded that since 10-hydroxy-2-decenoic acid is present in the food of larvae which become workers (since this acid was found in the mandibular glands of worker bees), it cannot be the queen-differentiating substance. Brown & Felaver (160) have suggested the possibility that the 10-hydroxy-2-decenoic acid might be a precursor to the queen substance (9-oxo-2-decenoic acid) to be discussed later in this review. They cited evidence that 9-hydroxy-2-decenoic acid which might be an intermediate in this conversion occurs in royal jelly in small amounts. The possibility that ACh or the ACh-complex of Henschler (153) is involved in queen differentiation has not been tested.

Claims have been made that ACh occurs in ciliated protozoa (*Paramecium*) (161), that AChE is present in another ciliate (*Tetrahymena pyriformis*), and that this enzyme is located in the basal region of the cilia where the fibrillar system is localized (162, 163). Acetylcholine-like activity was found in extracts of the motile *Trypansoma rhodesiense* but not in the nonmotile blood form of *Plasmodium gallinaceum* (164). In addition, eserine was reported to inhibit ciliary movement in *Tetrahymena* (162). These observations have led to the suggestion that ACh and its enzyme are implicated in the origin and co-ordination of the beat of cilia and flagella. The results are not entirely uncontested, however, since Tibbs (165) was unable to find AChE activity in homogenates of *Polytoma uvella*, *Polytomella caeca*, and in *Tetrahymena pyriformis*. In perch sperm, moreover, AChE activity was limited to the head, the tail region being negative. The cilia in the gills of *Mytilus edulis* were examined on the assumption, difficult to prove or to disprove, that the gill plate has no nerve fibers (166). Acetylcholine (10^{-6} to

[4] A paper seen only in abstract [*Bee World*, **41**, 279 (1960)] indicates the existence of a substance with biological and chemical properties corresponding to ACh in royal jelly (5 μg/mg). This substance was 1000 times more concentrated in royal jelly than in honey. Both honey and royal jelly were found to have substances hindering hydrolysis of ACh accounting for the stability of the ester.

10^{-5}) accelerated the rate of beat and the transport of particles. At higher concentrations (10^{-4} to 10^{-3}) there was reduction of both rate and transport. Eserine, too, showed a similar biphasic action according to concentration. Atropine acted like ACh but also reduced the response to ACh, whereas (+)-tubocurarine diminished both rate of beat and of transport. The action of epinephrine (10^{-6} to 10^{-4}) was an excitatory one and no slowing was observed. Extracts of the gill tissue examined by bioassay and paper chromatography showed evidence of an ACh-like substance. Acetylcholine esterase was also detected and Milton (167) obtained data indicating the occurrence of choline acetylase in the gill plates. An apparent correlation was described between the appearance and development of both AChE activity and co-ordinated ciliary movements in the developing larva of the sea urchin (*Paracentrotus lividus*) (168). Enzyme activity was first detected seven hours after fertilization. It increased slowly at first and then more rapidly to a high level at 50 hours of development. No nerve fibers are believed to be present in the early larva so that AChE appeared before any nerve or muscle tissue. On the other hand, the early phase of AChE growth was approximately coincident with the appearance of the ciliary movements and the formation of the larval ciliary bands with their intricate and co-ordinated activities. Lithium, which prevents development of the ectoderm, was also shown to slow the early phase of growth of the AChE system (168). The precise morphological localization of the AChE system in these echinoderm larvae, an important datum, has apparently not been determined.

Acetylcholine has also been conceived to be essential in initiating the excitation of the vertebrate heart (169, 170). Several investigators have noted that this substance was also able to regularize or strengthen the beat of an enfeebled invertebrate heart (126) or to produce a positive inotropic effect at very low concentrations (147). The idea of ACh as a local hormone required to maintain the beat was contested (148, 171) on the basis that ACh is, in fact, a waste product of metabolism produced by the beating heart and that the role of AChE is to destroy this toxic substance. No physiological role for ACh either as a local hormone or as a mediator was admitted by Jullien and co-workers (172, 173).

Noncholinergic Systems

Cephalopods.—Chemical mediation was suggested for the synapses of the squid stellate ganglion because (*a*) electrotonic currents failed to traverse the synapse, (*b*) the long synaptic delay of 0.4 msec, and (*c*) the synaptic delay was lengthened as temperature was lowered (174). This ganglion was considered to have both ACh and ACh-splitting activity (111), but attempts to demonstrate any responses to added ACh and related drugs were unsuccessful (111). Bacq was also unable to influence, with ACh, the responses to preganglionic stimulation, even after eserine treatment. Furthermore, there was no reduction of ACh-like activity in the ganglion ten hours after the preganglionic fibers were cut (111). More recently, Bryant (175) was un-

able to discover any significant physiological effects of ACh, eserine, (+)-tubocurarine, atropine, and certain other agents which have been employed in elucidating cholinergic and adrenergic mechanisms. The old objection of the existence of barriers can always be invoked to explain these negative findings, but at present this position cannot be evaluated. Electrophoretic micro-application of drugs should enable a clear decision to be reached. A similar tentative situation exists with regard to the neuromuscular systems of cephalopods. Though ACh excited the mantle muscle of *Eusepia* and this was antagonized by curare, the effects of nerve stimulation were uninfluenced either by curare or eserine (111). Bacq also perfused the mantle with eserinized sea water, and though ACh-like activity was present in the perfusion fluid, no increase in this activity was detected following motor stimulation. The present position is that a cholinergic mechanism for cephalopod synapses or myoneural junctions remains unproved. Cephalopods may not be the only molluscs for which this statement applies. The neuromuscular preparations of *Mya arenaria* and *Buccinum undatum*, for example, were found to yield no responses to eserine, to atropine, and to curare in the manner expected of cholinergic systems (108). The inhibitory nerve fibers to the cephalopod heart have sometimes been considered to mediate their effects by way of ACh. Such a view must be assessed with regard for the following experimental results: eserine did not prolong or increase the neural inhibitory process even though this AChE inhibitor was able to sensitize the heart to added ACh. Atropine was unable to block either the inhibitory or the excitatory neural actions, although curare prevented the former but not the latter of these two. These results (176, 177) obtained with squid and octopus are in agreement with Ransom's early data on *Octopus vulgaris* (178). An attempt was also made (179) to detect the appearance, following visceral nerve stimulation, of substances mimicking the neural effects on the octopus heart. The results were negative in an experiment employing a donor- recipient heart preparation mounted in a double Kahn cannula. Four experiments were made in which the perfusate collected during nerve stimulation was assayed on an eserinized sea cucumber muscle. Only one of these involved a positive result, and this effect was unlike that of ACh. The effect of caffeine in augmenting both neural inhibition and the action of ACh was demonstrated, but in the light of the other evidence, it is impossible to base an hypothesis of a cholinergic mechanism on this result.

The ascidian heart.—The tunicate heart is characteristically different from both the arthropod and molluscan hearts. The beat is prominently peristaltic; there is periodic alternation of beat origin from the two ends of the heart; there is probably no extra-cardiac neural control; and the pharmacology appears to be unique (180). No significant pharmacological alterations were produced by ACh, epinephrine, and ergotamine on the isolated heart of *Ciona intestinalis* (181, 182). This heart was restudied recently by use of a ligature which separated the visceral and hypobranchial pacemakers functionally and so avoided the complication of periodic reversal (183). Acetyl-

choline (10^{-6}) produced slight acceleration of beat from both pacemakers, and higher concentrations (10^{-3}) lowered the rate and decreased the amplitude of beat. Eserine neither had an effect of its own nor did it augment the excitatory or inhibitory actions of Ach. In addition, extracts of *Ciona* and of *Phallusia* gave no ACh-like reaction with the eserinized frog rectus abdominis and showed little ability to split ACh (65). Durante (101) saw no histochemical staining for AChE in the ascidian adult.

Finally, Bacq (112) obtained no potentiation by eserine of the contractions of the body-wall muscles as the result of electrical stimulation. The case has not been made therefore for cholinergic mechanisms in ascidians. If adult ascidians have no cholinergic mechanisms this presents an interesting biological problem in view of the occurrence of chordate taxonomic characteristics in this group of animals. However, in view of Durante's finding (101) of AChE in the tunicate larva, it may be that the cholinergic system was lost secondarily rather than never having been acquired.

Coelenterates.—Coelenterates posses a diffuse nerve net and are of special interest since they offer an opportunity to study a primitive and undifferentiated nervous system. The striking facilitation which is known to occur both within units of the net and between the net and the muscles (184, 185) offers an opportunity for comparative pharmacologists to investigate the possibility of the existence of transmitter substances as operants in the mechanism. Pharmacological data on these organisms are limited, but those which are available suggest neither a cholinergic nor an adrenergic system in coelenterates. Little or no ACh-like activity was recorded in a number of coelenterates so far examined (65, 186), and no ACh-splitting action by extracts of several species was noted (111, 187). Moreover, Bacq (111) was unable to record any contraction, even after eserine, of the isolated sphincters of *Calliactis* and *Metridium* in response to ACh (10^{-5} to 10^{-3}) though potassium did cause contractions.

Employing (*a*) the whole animal (188), (*b*) isolated rings from the column (189), and (*c*) sphincter preparations (190) of *Calliactes parasitica*, Ross was not able to record any significant actions by ACh, eserine, atropine, nicotine, (+)-tubocurarine, and tetramethylammonium (TMA). The column rings were excited to contraction by epinephrine, but cocaine did not potentiate this effect and several adrenergic blocking agents were ineffective in antagonizing either the responses to electrical stimulation or to epinephrine (189). Epinephrine evoked slow contractions (but not quick contractions) in sphincter preparations (190), but again the interactions with cocaine and adrenergic antagonists were negative (190). Not much of a case can be made for either a cholinergic or an adrenergic mechanism in these coelenterates. It is of general interest that ATP was able to produce contractions in the column preparations (189). Histamine, strychnine, and GABA were without effect. Of special interest were the actions of tyramine which, though inactive by itself, was able to facilitate the response to electrical stimulation (188). This was confirmed on sphincter preparations (190) in

which it was discovered that the enhancement involved quick but not the slow contractions. Of the indolylalkylamines which were tried, tryptamine was found to facilitate the quick contractions to electrical stimuli. 5-Hydroxytryptamine was much less active, while *N,N*-diethyl-D-lysergamide (LSD-25) and reserpine were inactive (189 to 191). These studies with drugs have therefore led to no firm hypothesis regarding the nature of possible transmitters involved in facilitation in coelenterates. Attempts to discover naturally occurring substances in coelenterates which might mimic the results of electrical stimulation are few, although Ross (192) found evidence in extracts of sea anemones for something which was able to facilitate the responses to single electrical stimuli. In certain respects the action of the extract was dissimilar to the manner of action of both tyramine and tryptamine. Certain pharmacologically active substances have been reported for coelenterates (5, 186, 193). These include histamine, 5-HT, TMA, homarine (α-picolinic acid N-methyl betaine), anemonine (imidazoleacetic acid dimethyl betaine), γ-butyrylbetaine, choline, and trigonelline (N-methylpyridinium hydroxide). Some of these substances may be components of the stinging organelles (nematocysts) employed in defense or predation.

The drug effects described by Ross (190) were probably not actions on sensory elements of the coelenterates. In contrast, a very specific chemosensory response of coelenterates (*Hydra littoralis*) is the feeding reaction elicited by small quantities (.001 mg) of reduced glutathione (GSH). This reaction is probably of biological significance since GSH may be the active substance released from prey stung by the penetrant nematocysts of *Hydra* (194). The feeding response to GSH is very specific. The following were ineffective in calling forth such a reaction: oxidized glutathione, S-acetylglutathione, γ-glutamyl-cysteine, cysteinyl glycine, γ-glutamylalanine, glycyl-cysteine, N-acetyl-cysteine, coenzyme A, glutamic acid, glutamine, cysteine, glycine, and ascorbic acid (194). Most interesting was the finding that asparthione (β-aspartylcysteinylglycine) did not work (195) implicating the γ-glutamyl moiety as partly essential for activity. The sulfhydryl group does not appear to be needed since ophthalmic acid (γ-glutamyl-α-amino-*n*-butyrylglycine) and norophthalmic acid both were active, the former even more so than GSH (196). A number of compounds, though inactive themselves, were inhibitors, apparently competitively, of the GSH reaction. These were glutamic acid, glutamine, asparthione, S-acetylglutathione, γ-L-glutamyl-L-sulphi-alanylglycine, and oxidized glutathione (195). These results suggest that specific configurational features are important in activity and that both the γ-glutamyl and glycine moieties are needed. *Hydra* may not be the only coelenterate which responds in this manner since *Physalia* was also discovered to give a feeding reaction to GSH (197). The receptors which respond in this way have not been identified, but other invertebrate receptors having a high degree of chemical specificity in their responses are known, for example, the antennal receptors of certain moths, which will be discussed later.

Parasitic flatworms.—Work on these organisms is very limited and there are only indications that pharmacologically they react differently to the drugs under consideration. The cat tapeworm (*Taenia taeniaeformis*) was employed to illustrate that epinephrine, histamine, and ACh were without significant action (198). After eserine, ACh, rather than causing excitation, stopped spontaneous activity and produced relaxation of tone. Atropine, hexamethonium, and (+)-tubocurarine were unable to block this action of ACh. Benzoylcholine, in contrast, produced effects on tone (different for the intact piece and the strip with lateral portions sliced off) which were antagonized by hexamethonium. 5-Hydroxytryptamine was excitatory for both the tapeworm and liver fluke (198, 199), but the effective concentration ($2.5 \times 10^{-5} M$) was much higher than is usually required for pharmacological actions by this substance. LSD-25 ($5 \times 10^{-9} M$) had an inhibitory action on the liver fluke but higher concentrations ($10^{-7} M$) caused increase in tone and rhythmic movements. Bromolysergic acid diethylamide had an inhibitory effect by itself but, in addition, was able to block the excitatory actions of both LSD-25 and 5-HT. Epinephrine, norepinephrine, and histamine were ineffective on the liver fluke.

Crustacean myoneural junctions.—Crustacean neuromuscular systems offer special opportunities to examine in the periphery certain characteristics which, in vertebrates, are important to functioning of the central nervous system. Facilitation (200, 201), inhibition (201, 202), and the consequences of multiple innervation (203, 204) are all clearly displayed in these crustacean preparations. It is generally assumed that the effects of the skeletal muscle nerves are mediated by way of chemical substances (28, 201). It has not been an easy matter to prove this or to identify the mediators. There is almost general agreement that ACh is not one of the mediators. Though ACh-like activity was found in nervous tissue of crustaceans, muscle tissue appears to be singularly free of such action (34, 65, 130).

Acetylcholine-splitting activity was originally thought to be absent in crustacean muscle (82), but later work reversed this decision (111, 187). No success has been achieved in attempts to demonstrate pharmacological effects on crustacean muscle by added ACh (82, 205, 206). Katz (201) assayed the effects of ACh, with and without eserine, and was unable to detect any improved facilitation. Except for some evidence of reversible neuromuscular block (207), many investigators have reported only negative results for curare and similar drugs (201, 206, 208). Other drugs have also been tried but have not significantly narrowed the search for the suspected transmitters. Strychnine was observed to be without effect on the lobster neuromuscular membrane (208). Other investigators have found nicotine and pilocarpine to be ineffective (206), although evidence of some excitatory response to epinephrine has been obtained (201, 205, 206). Recent investigations have introduced GABA into crustacean pharmacology. Confirming some preliminary work (209, 210), GABA was reported to inhibit or prevent contraction of the opener muscle of the crayfish claw, a muscle pro-

vided with dual innervation via an excitatory and an inhibitory nerve (211, 212). Picrotoxin was shown to act reversibly in antagonizing the effects of both GABA and inhibitory nerve stimulation (204, 212, 213). In contrast, picrotoxin neither enhanced, nor antagonized the contractions elicited by excitatory nerve stimulation nor did it interfere with conduction in the inhibitory nerve fibers (204). A specific effect at the nerve-muscle junction of the inhibitory system was suggested. Boistel & Fatt (214) concluded that both the hypothetical transmitter released by inhibitory nerve stimulation and GABA increased membrane conductance. They visualized the inhibitory action in these crayfish muscles as involving an increased permeability to Cl^-, though K^+ could not be eliminated entirely. In another study of the lobster neuromuscular system (208), GABA was shown to increase membrane conductance, an effect which was rapidly produced, readily reversed, and evident even at GABA concentrations of 10^{-12}. GABA also acted, as did inhibitory nerve stimulation, in depressing the excitatory postsynaptic potential elicited by the excitatory nerve fiber. Picrotoxin blocked the inhibitory action (the inhibitory postsynaptic potential and the change in conductance) even though alone it had no action on the membrane conductance or the excitatory postsynaptic potential. Picrotoxin also antagonized the membrane changes produced by GABA. In all these responses, GABA mimicked in a convincing fashion the actions of the inhibitory nerve fiber, but this amino acid was not alone in these actions since β-alanine, β-hydroxy-GABA, γ-aminocrotonic acid, and guanidoacetic acid all increased membrane conductance without evidence of depolarization. The similarity of GABA to the inhibitory transmitter may be more apparent than real since Van der Kloot (215) published some data in support of the idea that GABA acted by causing a release of the unidentified mediator. Recently (216) it was suggested that GABA may act on the motor nerve endings, possibly reducing the output of excitatory transmitter.

In comparison with the inhibitory junctions, the crustacean excitatory junctions appear to have different pharmacological properties. L-Glutamate in relatively low concentrations is known to have reversible excitatory effects (211, 217, 218). Applied externally to the skeletal muscles of *Cambarus clarkii*, this amino acid caused a quick contraction and a depolarization of the muscle membrane (218). After removing the glutamate and washing the muscle, there were after effects which persisted for a few minutes. These consisted of a depression of both the contractions and action potentials to indirect stimulation, although no such depression was observed in the responses to direct stimulation. Glutamate was inert with respect to the nerve fibers. These results suggested an action at the excitatory junctions. The glutamate excitatory action was highly specific. With *Cambarus* preparations only L-glutamine showed activity, but this was much less active than was L-glutamate and a conversion to glutamate might have occurred (218). Other compounds such as D-glutamate, L-aspartate, L-α-aminoadipate, L-asparagine, glutarate, succinate, L-ornithine, L-proline ($\pm$)-α-amino buty-

rate, α-ketoglutarate, and carnitine were either inactive or were required in much higher concentrations. The excitatory action of L-glutamate was antagonized by both inhibitory nerve stimulation and by GABA (218). For example, the means of ten trials showed the threshold concentration for L-glutamate alone to be $3.8\times10^{-5}M$, for L-glutamate plus L-aspartate the threshold was $3.8\times10^{-5}M$, for L-glutamate and GABA the threshold was $1.7\times10^{-4}M$.

The effects of GABA and glutamic acid on crustacean myoneural junctions show both similarities and differences when compared with the results on the cat spinal cord in which electrophoretic local application of several amino acids was used while studying activity of interneurones, motoneurones, and Renshaw cells (219, 220). Glutamate, aspartate, and cysteate were found to be effectve depolarizing and excitatory substances. L- and D-Glutamate were of about the same effectiveness. The cholinoceptive Renshaw cells were also excited by glutamate, but β-erythroidine did not block this excitatory action. GABA and β-alanine were not excitatory but inhibitory, but this inhibitory action did not involve, as in the crustacean myoneural junction, a shift in membrane potential (221, 222) Strychnine, the antagonist of inhibition of the spinal cord, did not prevent the actions of GABA and β-alanine. These results suggest the occurrence of interesting pharmacological interactions of a general nature rather than specific interventions with transmitter mechanisms. A calcium mechanism may be involved in the actions of the dicarboxylic acids.

The crustacean stretch receptors.—The receptors discovered by Alexandrowicz (53) in the abdomen and thorax of crustacea have offered readily accessible cells for the study of several basic problems in physiology and pharmacology. The existence of both excitatory and inhibitory mechanisms at the dendritic terminals of these cells has made them ideal models for the study of interacting neuronal activity. Acetylcholine was able to excite these cells: eserine potentiated this effect, whereas atropine proved to be a reversible antagonist (223, 224, 225). Atropine, however, did not block the normal response to stretch (223). Good reasons were given for believing that the ACh effect was a direct one on the receptor rather than an action via the muscle fibers. Excitation was also observed with 5-HT, an effect which was abolished by tryptamine (225). The particularly intriguing property of the crustacean abdominal stretch receptors is their innervation with accessory nerve fibers which are efferent in function. In both lobster and crayfish, stimulation of these was shown to lead to inhibition of the sensory discharge of the receptor (226, 227).

GABA is capable of producing rapid and reversible inhibition of the stretch receptor discharge (228, 229) and to interact with the effects of the inhibitory nerve stimulation (229). The interpretation was made that GABA caused a specific conductance increase in the membrane of the dendrites and cell body but not of the axon (229). The excitatory effects of ACh, 5-HT, and nicotine on the crayfish stretch receptor were also depressed

by GABA (225). Though GABA was most active in blocking sensory excitation, it was by no means the only substance capable of so acting (225, 228, 229). The structural features which appeared to be especially significant in inhibitory action were: the presence of positive (NH_2) and negative (COOH) groups separated by three carbons and the absence of neutral groups in the carbon chain. Guanido acids (guanidoacetic, β-guanidopropionic) were almost as effective as GABA (229). Of some interest was the finding (230) that γ-aminobutyrylcholine was inhibitory (though less effective than GABA); butyrylcholine, on the contrary, was excitatory. By stirring the GABA solution around the receptor and re-establishing a block of discharges, Edwards & Kuffler (229) obtained an indication that some inactivation or removal of GABA occurred in the preparation. Picrotoxin was effective in blocking the GABA inhibition of this receptor, but it was by no means specific in this respect since atropine, tryptamine, Dibenamine, LSD-25, and metrazol were also effective but in varying degrees (225).

These experiments with GABA need not be interpreted as supporting the hypothesis that GABA is the inhibitory transmitter. We now know three apparently different types of reactions in invertebrates: (*a*) inhibitory junctions in which GABA mimics the membrane effects of inhibitory nerve stimulation (crustacean stretch receptors and myoneural junctions), (*b*) inhibitory junctions in which GABA is without apparent effect (the *Onchidium* ganglion), and (*c*) nerve cells without inhibitory prefibers in which GABA causes membrane hyperpolarization and discharge inhibition (Tauc's D-cells in *Aplysia*). The evidence as it now stands cannot logically support GABA as the inhibitory transmitter.

Catechol Amines

The possible presence in annelids of cells with chromaffin granules and of epinephrine in the ventral nerve cord was indicated by investigators early in this century [cited by Perez (231)]. Following up these suggestions, Gaskell (104, 232) examined the nerve cells in the ventral nerve cord of the medicinal leech and observed the results of cutting and of stimulating the processes from these cells on the rhythmic contractions of the lateral vascular vessels. It was concluded that these vessels are under control of the central nervous system and provided with dual innervation, one set of fibers probably originating in the chromaffin central nerve cells, passing via the anterior segmental nerves and mediating an excitatory control of the muscles, with the second system of fibers passing via the posterior segmental nerves and exerting inhibitory control on the vascular vessels. Curare abolished the inhibitory effect whereas atropine accelerated the beat. An extract prepared from 400 leech ganglia and tested on the histamine-stimulated cat uterus gave inhibition similar to the action of epinephrine. The occurrence of an epinephrine-like substance in extracts of *Hirudo* was confirmed by Wense (233) who found in the extract a labile substance which excited the atropinized frog heart and inhibited the movements of the rabbit

intestine, an effect which was potentiated by cocaine. Norepinephrine was detected in earthworm extracts by von Euler (234). Although no effects of epinephrine on annelid vascular vessels have been reported by some workers (235), others were able to confirm Gaskell's finding of an excitatory action (236). Gaskell, in fact, postulated that muscular vascular vessels and chromaffin cells were present in some annelids and absent in others. Gaskell's important generalization was that in annelids a single cell type is the precursor of both the vertebrate sympathetic nerve cell and the chromaffin cell of the adrenal medulla. This conclusion was supported by Lancaster (237) who observed the presence of chromaffin cells, not only in the nerve cords of leeches and the earthworm, but also in the crayfish and the grasshopper. Perez (231) obtained true chromaffin staining in two cells of the anteromedian follicle of typical ganglia of the leech. While admitting that the chromaffin cells are probably nerve cells, Perez chose to disregard these as forerunners of the vertebrate sypathetic system and concluded, as had Vialli before him (cited by Perez), that the leech already has a well-developed separate vegetative nervous system. Perez viewed the chromaffin cells as secretory cells, ancestral to the system in the adrenal medulla. Gaskell's suggestion of dually innervated visceral organs is interesting in the light of Wu's observations (238), on the gut (crop portion) of the earthworms *Lumbricus* and *Allolobophora*, that stimulating the circumpharyngeal commissures caused excitation, as did ACh, whereas nerve-cord stimulation led to inhibition of spontaneous movements, as did epinephrine. Moreover, atropine blocked the effect of commissural stimulation while ergotoxine prevented the nerve cord mediation. A cholinergic-adrenergic duality similar to that in vertebrates was suggested (238, 238a).

Gaskell's conclusions and the known effects of ACh on the leech body wall prompted Pantin (239) to suggest that cholinergic and adrenergic mediator mechanisms were not unique to vertebrates but had developed early in evolution and probably occurred in many coelomate invertebrates. We have already recounted the situation with regard to cholinergic systems. The status of the adrenergic question is even more uncertain. A substance with epinephrine-like properties was detected in extracts of *Paramecium* (240) and in extracts of the purple gland of molluscs (241). Erspamer (152) observed that extracts of the posterior salivary glands of *Octopus vulgaris* acquired epinephrine-like properties after irradiation with ultraviolet light. The properties of the unknown precursor were consistent with an amine having a free phenolic group. This substance, octopamine, was later identified as 1-(*p*-hydroxyphenyl)-2-aminoethanol (242), and the product of irradiation was identified as norepinephrine. Octopamine may be involved in the biosynthesis of norepinephrine which was detected in extracts of the posterior salivary glands of *Octopus vulgaris* (243). Extracts of the glands of *Octopus macropus* gave lower values for the quantities of both octopamine (152) and norepinephrine (243). A few attempts were made to assay the quantity of catechol amines in the tissues of other invertebrates. Little or no activity

was noted for protozoa, coelenterates, echinoderms, molluscs, crustaceans, and tunicates (243, 244). Annelid worms and the posteror salivary glands of certain cephalopods appear to be unique in their content of these substances. Data on insects are very scarce, but Wense (233) obtained crystals from extracts of the mealworm larva which, chemically and pharmacologically, behaved as did epinephrine. On repetition of this work, Gregerman & Wald (245) were unable to detect any epinephrine, although as little as 250 μg of authentic epinephrine added to 35 g of larvae at the beginning of the extraction procedure was recoverable as a spot on paper. Evidence was obtained, instead, of two spots, probably *o*-diphenols, but neither of these was epinephrine or DOPA. Supplementing his bioassay analyses with paper chromatography, Östlund (244) concluded that DOPA in quantities up to 15 μg/g occurred in various insects, that norepinephrine (.05 to 2.2 μg/g) also occurred, but that epinephrine was present in much smaller quantities (0.01 to 0.3 μg/g). This amount of epinephrine was considered to be below the value detectable by Gregerman & Wald's methods. Östlund also observed an unknown spot, thought to be a catechol amine, which he called catechol-4. Cameron (246) obtained an extract from the corpora cardiaca of the cockroach (*Periplaneta*) which was pharmacologically active on the heart and gut of this roach. Paper chromatograms of extracts of mealworm larvae revealed, in addition to Gregerman & Wald's two spots, a third spot which after elution showed the pharmacological activity of corpus cardiacum extracts. This active substance, found also in extracts of cockroach, was not epinephrine; Cameron concluded the "insects do not secrete adrenaline, but another orthodiphenol having the functions of adrenaline in the insect body." There is little doubt that many insects have *o*-dihydric phenols (247, 248). One suggested function for some of these substances is as participants in the tanning reaction of the cuticle and the oötheca (249, 250). Tissue extracts of many invertebrates oxidize amines, including epinephrine (251). Amine oxidases have been found in echinoderms (252), in some molluscs (252 to 255), and in the earthworm (256). They have not been found in all invertebrates in which they were sought (254, 256, 257). The physiological significance of this enzyme system is still in doubt, and it may well be that it is simply involved in preventing the accumulation of toxic amines rather than in the more specific role of inactivating catechol amine transmitters (258, 259).

Pharmacological actions of catechol amines.—The literature concerned with the invertebrate heart is difficult to evaluate since experimental conditions have varied and since the hearts themselves are so diverse anatomically and otherwise (120 to 123, 180). Certain very general statements may be made, however, in order to guide the reader through this maze of descriptive publications. Epinephrine in low concentrations was reported to excite the arthropod heart (121). Carlson (260) observed the excitatory effects of epinephrine on both the cardiac ganglion and the ganglion-free portion of the *Limulus* heart. This was confirmed by other investigators

(261, 262, 263). The crustacean heart is also excited by epinephrine (135, 264, 265). Observed effects of epinephrine were increases in rate, tone, and amplitude (265). Ergotoxine, by itself, was a depressant but in no case was it able to antagonize the excitatory role of epinephrine. In fact, a heart completely stopped through the influence of ergotoxine could be restored to regular boat by epinephrine. Pilocarpine had an excitatory action similar to that of epinephrine, but unlike epinephrine its action was blocked or reversed by atropine. Welsh (131) compared the excitatory actions of ACh and epinephrine on the heart of the spiny lobster. He considered the points of attack of these two drugs to be different since ACh in higher concentration caused systolic standstill, whereas epinephrine in higher concentration stopped the beat in diastole. Though some differences exist in the literature (128, 266), the general conclusion for the arthropod heart is that epinephrine at low concentrations causes reversible excitation, effects which appear to be similar to those of ACh though the point of attack, about which nothing is known, may be different for the two drugs.

The molluscan heart shows more diversified responses to catechol amines. One effect of epinephrine is an excitatory action. Bacq (142) obtained positive inotropic and chronotropic actions by epinephrine in low concentrations (10^{-9}) with the heart of *Loligo*. Tyramine caused similar excitation but was 100 to 1000 times less active. Ergotamine by itself produced stimulation but additionally blocked or diminished the responses to epinephrine. Excitatory actions of epinephrine have been reported for other cephalopod hearts (244, 267, 268, 269). The hearts of some lamellibranchs and gastropods also reacted to epinephrine by acceleration and augmentation of beat (38, 42, 123, 140), but in other species there were either very small or no effects of epinephrine in pharmacological concentration (42, 141, 147, 270) or else inhibitiory responses were recorded (271). A striking demonstration of the differences in various molluscs is illustrated in the experiments of Erspamer & Ghiretti (268) in which it was noted that as little as 0.1 μg of epinephrine stimulated the octopus heart whereas as much as 15 μg had no effect on the heart of *Helix pomatia*. A multiphasic action of epinephrine according to concentrations was obtained with the heart of *Venus* (271). At threshold concentrations of both epinephrine and norepinephrine (10^{-5} to 5×10^{-5} M) a small but definite negative inotropic action was obtained. A small increase in concentration (2×10^{-5} to 7×10^{-5} M) produced a positive chronotropic response along with the negative inotropic action. Still further increase (7×10^{-5} to $12^{-10^{-5}}$ M) resulted in a marked rise in tone along with systolic arrest. These results are striking in the rapid qualitative changes which occurred with only minor increases in dose of drug. Such a series of effects could easily have been missed by other investigators of the molluscan heart. Greenberg considered that the initial negative inotropic response was not an ACh effect (released by epinephrine, for example) since benzoquinonium, an ACh antagonist, was employed.

Another unique feature of the *Venus* heart was the sensitivity to dopa-

mine which was about ten times greater than to epinephrine and norepinephrine. This is in contrast to the cephalopod heart (269) for which dopamine was much less active than either epinephrine or norepinephrine. The response of the *Venus* heart to catechol amines was different in several respects from the action of 5-HT. On a molar basis 5-HT was much more active. Threshold concentration was $10^{-9}M$ and between this value and $10^{-6}M$ the response was chiefly a positive inotropic action. At higher concentration (up to $10^{-5}M$) there was an increase in tone. The 5-HT effect, unlike the actions of catechol amines, was antagonized by bromolyspergic acid diethylamide (10^{-5} g/ml). Curiously, the responses to tyramine and to phenethylamine (10^{-5} to $10^{-4}M$) were 5-HT-like and were blocked by bromolysergic acid diethylamide.

5-Hydroxytryptamine and Other Indolylalkylamines

The history of the discovery of 5-HT as a substance of natural occurrence (272) includes an important chapter involving the invertebrates. Enteramine, now known to be 5-HT, was found in the alimentary tract of two ascidians, *Tethium plicatum* and *Ciona intestinalis* (273). Enteramine-like activity was also noted in extracts of the posterior salivary glands of *Octopus vulgaris* and *Eledone moschata* (274) and in the hypobranchial glands of prosobranch molluscs (275). The picrate of enteramine was prepared from extracts of the posterior salivary gland of *Octopus vulgaris*. This, after recrystallization, moved as a unit in paper chromatography. The properties (melting point, empirical formula, ultraviolet absorbtion spectra in alkaline, and acid solutions) indicated that octopus enteramine was identical with the serotonin (276, 277) to which Rapport assigned the structure of 5-HT (276, 277). Synthetic 5-HT was soon available (278, 279) and this was found to agree chemically and pharmacologically with serotonin (278, 279) and with octopus enteramine (276). The content of 5-HT in the salivary glands of cephalopods varies considerably from species to species. It was absent, for example, in extracts of the posterior salivary glands of *Octopus macropus* and the anterior salivary glands of *Octopus vulgaris* (274, 275). 5-Hydroxytryptamine appears to be present in many, but not in all invertebrates (52). Negligible quantities were noted in the amoeba, *Pelomyxa*, and the sponge, *Halichondria*. These data from animals lacking neurones need to be supplemented by more analyses from several protozoan and sponge classes. Significant quantities of 5-HT were found in coelenterates, but whether or not this substance is anatomically localized in the nerve net or in other structures is not yet known.

5-Hydroxytryptamine was also found in Platyhelminthes and in Nemerteans where some cephalization of the nervous system occurs. In several annelid worms and a sipunculid, where it was possible to remove and isolate the nerve cords for analysis, there was evidence of a concentration of 5-HT within nervous tissue. Large concentrations of 5-HT were discovered in the nervous tissue (especially the ganglia) in the three classes of molluscs:

Amphineura, Pelecypoda, and Gastropoda. The highly active and well-differentiated cephalopods had much less 5-HT in the nervous system. In *Octopus vulgaris*, for example, though extracts of the posterior salivary gland showed 5-HT values of 68 to 72 μg/g fresh tissue, the "brain" had a value of 0.8 μg/g; the "brain" plus stellate ganglion, a value of 0.56 μg/g and the optic ganglion, a value of 2.3 μg/g. These values may be compared with the figure of 40 μg/g obtained from extracts of the ganglia of *Venus mercenaria*. The nervous system of arthropods showed little or no 5-HT but large quantities were found in the venom apparatus (scorpion, Hymenoptera), in the pericardial organs (crustacea) and in the excretory organs (certain crabs). 5-Hydroxytryptamine was found in the very ancient arthropod, *Limulus*, in which a relatively high concentration was present in the excretory organ, the coxal gland. The echinoderms were found to have little, if any 5-HT in their tissues.

The association of a large quantity of 5-HT with venomous organelles or organs has been indicated by several investigators. The nematocysts of coelenterates have been claimed to be sources of 5-HT (280), but this was denied (193) for the reason that the tentacles of *Calliactis* had little 5-HT and that little or none was found in *Metridium*, *Anemonia*, or *Physalia*. Phillips & Abbott (281) isolated nematocysts and freed them of tissue components and algae. Though 5-HT was detected in these isolated nematocysts, the amount became less as more and more tissue components were removed from the suspensions. Incidentally, bufotenine (*N,N*-dimethyl-5-HT) was also detected in the tissues of some coelenterates (280, 282). 5-Hydroxytryptamine was found in the saliva of *Octopus vulgaris* (283) and, along with tyramine, octopamine, histamine, ACh, taurine, and dopamine, in the salivary glands of certain cephalopods (284, 285). The venoms of the scorpion (*Leiurus*) and of the wasp (*Vespa*) were found to contain 5-HT (286, 287). These venomous secretions have, of course, many different components (5) and the role of 5-HT, if any, in the action of the venom is not always clear. Delayed, but prolonged pain has been reported to occur in man by solutions (10^{-8}) of 5-HT (288) and, in addition, 5-HT, like other amines, can act as a histamine releaser (289). The possible biological role of this indole in defensive reactions is also suggested by its occurrence in the skin of certain amphibia and in the stinging nettles of certain plants (290).

Finally, it is of some interest to point out that 5-HT may be released, in invertebrates as in vertebrates, by reserpine administration (291). This was demonstrated in *Octopus vulgaris* and *Eledone moschata*, the significant feature being that whereas the 5-HT content in the optic ganglion dropped almost to zero after reserpine, that of the posterior salivary gland was unchanged.

The molluscan heart is accelerated by 5-HT in low concentrations, and this substance has been suggested as a possible mediator of cardioaccelerator nerve action (146, 292). The evidence for such a role is not convincing but the molluscan heart in relation to 5-HT has revealed some striking similar-

ities to the pharmacological behavior of the vertebrate brain and other vertebrate organs relevant to 5-HT. Reversible augmentation (amplitude, frequency, tone) of the molluscan heart beat was demonstrated (268, 275), and it was shown that this action was not comparable to that of epinephrine since for some molluscs (*Helix*) epinephrine did not cause excitation whereas 5-HT did. The similarity between 5-HT action and the results of nerve stimulation was insufficient to warrant consideration of this compound as a mediator of nerve action in cephalopods (293). Welsh (40) then demonstrated that 5-HT was far more effective in exciting the *Venus* heart than were epinephrine, norepinephrine, tyramine, and histamine, all of which gave augmentation of beat. The isolated ventricle of *Anodonta cygnea* showed positive inotropic and chronoptropic responses to 5-HT at concentrations as low as 10^{-9} g/ml and to respond over a concentration range of 3 log units (294).

Other drugs had (*a*) no clear effects (tyramine, dopamine, substance P, histamine, ACh) (*b*) positive inotropic actions with relatively high concentrations (dihydroergotamine, cocaine), or (*c*) negative inotropic actions (epinephrine, norepinephrine). Lysergic acid diethylamide was also found to be a potent exciting agent for the *Venus* heart (295, 296) whereas 2-bromolysergic acid diethylamide was able to reduce or block the responses to either 5-HT or LSD-25 (296). Recent investigations (271, 297) of the *Venus* heart not only confirmed some of these findings but added some new information. Prolonged exposure to relatively strong solutions of 5-HT ($10^{-5}M$) caused a loss of sensitivity to this compound, a tachyphylaxis similar to that produced in the guinea pig ileum (298, 299) but developing more slowly and not reversed by washing. Such desensitized hearts still responded to the catechol amines, and treatment with high concentrations of 5-HT ($2\times10^{-5}M$ to $10^{-4}M$) caused a decrease in amplitude of beat and a systolic arrest. These results suggested the existence of specific 5-HT receptors different from the sites of action for the catechol amines.

An examination of various structural analogues (297) indicated that the requirements for effective 5-HT-like action in the *Venus* heart were rather specific, consisting of an indole ring with a 2-aminoethyl side chain at position 3 and a hydroxyl group at position 5. The following structural changes all reduced activity of the compound: removal of the indole group or substituting a phenyl group in its place; removal of the side chain at position 2 or changing its length; removal of the $-$OH at position 5, substitution at position 2. Some of these requirements are similar to those which Vane (259) discovered for the rat fundus preparation. Thus, shortening the side chain (as in 3-indolymethylamine) or lengthening it [as in 3-(3-aminopropyl) indole] decreased the activity in comparison with tryptamine. For some compounds the results on the rat fundus and the *Venus* heart were apparently different. For example, α-methyltryptamine was much more potent than tryptamine on the rat fundus but the two compounds were equally active on the *Venus* heart. Vane explained the difference between the behavior of

these two substances on the fundus on the basis that whereas tryptamine is oxidized by amine oxidase, α-methyltryptamine is not. After inhibition of this enzyme the results on the fundus and *Venus* preparation were comparable, in accord with the assumption that there had occurred no oxidation of tryptamine by the *Venus* heart. This appears to be a justifiable assumption since Greenberg (297) was unable to demonstrate amine oxidase activity for the *Venus* heart. Some differences between the fundus and *Venus* preparations are not explainable in this manner. Bufotenine, for example, was much more active on the *Venus* heart than was 5-HT, whereas on the rat fundus it was about one-tenth as active as 5-HT; this ratio was little altered by inhibition of the amine oxidase.

The pharmacological actions of 5-HT on other invertebrate systems have not been examined sufficiently to permit comprehensive statements or safe conclusions. Little is known about the responses of arthropod hearts to this substance although there are indications that the crustacean heart is excited by 5-HT (300). The rhythmic electrical bursts of activity of the isolated cardiac ganglion of *Limulus* were slowed or abolished by 5-HT, an effect which was blocked by 2-bromolysergic acid diethylamide (301). The ability of 5-HT to accelerate relaxation of the *Mytilus* byssus muscle has already been mentioned in this review (113). 5-Hydroxytryptamine was also reported to antagonize the excitatory actions of ACh and nicotine on the dorsal body-wall muscle of the medicinal leech and to accelerate relaxation after these substances were washed out (302). Ciliary activity was observed to decrease in *Mytilus* gill filaments when the branchial nerve was cut from its origin, the visceral ganglion (303). Aqueous extracts of gill tissue as well as 5-HT and veratrine sulfate were able to reactivate cilia in excised tissue. Since 5-HT-like activity was found in these extracts, Aiello concluded that the branchial nerve mediates its action by release of 5-HT or a similar substance, but no information was presented as to the results of branchial nerve stimulation on ciliary activity. In addition, homogenates of *Mytilus* gill plates were shown to be able to oxidize 5-HT and other 5-hydroxyindoles (304). The implication of these results is that a 5-hydroxyindole is involved in control of ciliary activity in this mollusc. 5-Hydroxytryptamine has also been implicated in the activity of the pericardial organ of crustaceans. Extracts of these neurohaemal organs were shown to be able to accelerate the crustacean heart (305), though the effects were not interpretable in terms of a single substance. Florey & Florey (306) suggested that the active substance might be 5-HT, but Carlisle (307) was unable to discover a spot with an R_f value like that of 5-HT. Maynard & Welsh (300) confirmed the presence of a cardioaccelerating substance in extracts of pericardial organs but noted differences in action between the extract and 5-HT. Much of the activity was destroyed by trypsin or chymotrypsin but the active material was dialyzable. The few properties given for the active substance (300) are not unlike those of substance P, a polypeptide extracted from the intestine and nervous system of vertebrates (308). Physiologically active polypeptides have been discovered in several invertebrates. Jaques

& Schacter (287), for example, showed the presence in wasp venom of a substance, in addition to histamine and 5-HT, causing delayed slow contraction of the guinea pig ileum even after treating the ileum with atropine and mepyramine and after tryptamine desensitization. The activity was therefore not attributable to ACh, histamine, or 5-HT. The active substance was dialyzable and was inactivated by alkalinity, trypsin, or chymotrypsin (309). It was suggested to be a polypeptide with properties similar to those of bradykinin.

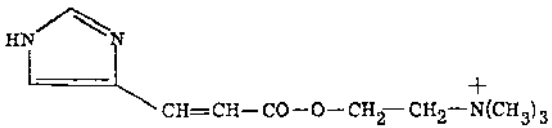
COMPOUNDS OF INVERTEBRATE ORIGIN

Under this heading we shall be concerned with a selected series of substances of pharmacological interest. Some of these are physiologically significant compounds with specific functions for the organism producing the material. Others are not known to serve any function but are of interest because of their relation to certain well-known drugs or to specific pharmacological problems. Many of these are unique to invertebrates and offer very special insight into broad biological problems of adaptation and adjustment to particular environments.

Choline Esters other than ACh

As in the vertebrates, choline esters other than ACh have also been found in certain invertebrates. The functions of these esters, if any, are quite unknown. In general they have not seriously interfered with the assays for ACh since these are usually found in unusual locations not directly associated with the nervous or neuromuscular system. They should be taken into account, as has sometimes not been done, in gross assays, for example, of the whole animal or the entire head. One source of these esters is as components of secretions produced by certain invertebrates.

Murexine (urocanycholine).—The hypobranchial glands of certain marine gastropods in the family Muricidae produce, in addition to a number of other substances, a choline ester of urocanic acid [β-(4-imidazolyl)-acrylic acid]. known as murexine:



The paralyzing and toxic actions of extracts of these glands on amphibia, fish, and certain invertebrates has been known for some time (310, 311). Extracts of the glands of *Murex trunculus* assayed on the leech muscle showed ACh-like action (312). Erspamer & Dordoni (313) next demonstrated that the substance in such extracts was unlike ACh in respect to its behavior toward AChE, atropine, and chemical treatment. After alkaline hydrolysis, which destroyed the pharmacological activity, and acetylation

of the product, activity indistinguishable from that of ACh was detected. An ester of choline, not ACh, was suspected and given the name murexine. The picrate, picrolonate, styphnate, flavianate, and reineckate were prepared (314). The elementary analyses of the purified compound and its derivatives and the nature of the acid were next determined. From this information, murexine was considered to be β-(4-imidazolyl)-acrylylcholine (315). Synthesis of urocanylcholine was next achieved and the elementary analysis, melting point, ultraviolet absorption, infrared spectrum, and pharmacological properties of the synthetic ester all indicated its identity with the natural substance (316, 317).

The pharmacological properties of murexine (318, 319, 320) involve both a ganglion stimulating and a neuromuscular blocking action in vertebrates, the latter being of the depolarizing type. (+)-Tubocurarine was shown to be an antagonist of murexine. There is general agreement that AChE cannot hydrolyze murexine, but that other tissue cholinesterases can do so (320, 321, 322). To the authors' knowledge, murexine has not been reported to be present in vertebrate tissues, though there is a note to the effect that imidazoleacetylcholine was found in extracts of the mammalian brain (323). A number of imidazole derivatives related to murexine have been synthesized (324) and compared pharmacologically with murexine (325). In studies of certain imidazole esters and imidazole ethers of choline, the following general principles of action emerged: hydrogenation of the acrylyl side chain into propionyl (as in dihydromurexine) caused an increase of both the nicotinic and neuromuscular blocking actions and of the effect on frog rectus. In man, however, dihydromurexine was less active than murexine as a muscle relaxant. This may be the result of the fact that human plasma cholinesterase splits dihydromurexine at a faster rate than murexine (321, 322). Maximal blocking and nicotinic actions were obtained with a three-carbon atom side chain for the imidazole acid (325). Imidazolecarboxylcholine and imidazoleacetylcholine, on the one hand, and imidazolebutyrylcholine, on the other, were all less potent than dihydromurexine. Substitution of an ether for an ester linkage led to either increase in blocking action (methoxy compared to carboxyl derivative) or to a small decrease (propoxy compared to propionyl). These results may lack generality in the sense that similar ratios of activity do not apply, as already indicated for man, for all species. Variations in blood and tissue cholinesterases, as well as other factors, may account for these variations.

Senecioylcholine (β,β-dimethylacrylylcholine).—From the hypobranchial gland of *Thais floridana* (Muricidae) the choline ester of senecioic acid was obtained (151). The presence of a new compound was first indicated by the appearance in column chromatography of a peak not present in extracts of other Muricidae; the substance had an absorption peak, not at 270 mμ (homarine), but near 230 mμ. It was pharmacologically and chromatographically different from ACh and propionylcholine, but a product of its hydrolysis was choline. The purified ester was found to have an absorption

band at 222 mμ suggesting an α,β-unsaturated fatty acid. The free acid was shown to have one double bond and hydrogenation resulted in the production of isovaleric acid. These observations identified the unsaturated acid as β,β-dimethylacrylic acid (senecioic acid). Finally, the ester was identified with authentic senecioylcholine by means of pharmacological and physicochemical behavior (326).

This choline ester may also occur in the prothoracic glands of the aposematic moth, *Arctia caja*, which produces a painful sting on handling, though the venom apparatus is not known (327). The pharmacological properties of senecioylcholine are not unlike those of murexine (328). Respiratory stimulation, probably the result of exciting the carotid sinus receptors, was noted, as well as excitation of sympathetic ganglia and neuromuscular block. Senecioylcholine was slowly hydrolyzed by human plasma cholinesterase but little, if at all, by AChE.

Acrylylcholine.—The hypobranchial gland of the nondye-secreting gastropod *Buccinum undatum* (Buccinidae), yielded the parent compound of the

previous two esters (329). This ester was identified by means of the same procedure and rationale as used for senecioylcholine. In this case an acid was recovered by steam distillation of the hydrolysate which could not be separated from authentic acrylic acid by vapor phase chromatography. Acrylycholine was synthesized and proved to be indistinguishable from the natural compound in column chromatography. This ester was reported to have both nicotinic action and neuromuscular blocking ability (330).

Other Compounds Interfering with Neuromuscular Transmission

A number of other substances of invertebrate origin, not all chemically characterized, have been implicated in neuromuscular interference in vertebrates. One of these is the very toxic substance which causes paralysis (and sometimes death) in man after ingestion of certain lamellibranchs (mussels, clams, scallops). The marine dinoflagellate, *Gonyaulax catenella*, has been implicated at least in the case of intoxication by the mussel, *Mytilus californianus*. The dinoflagellate has been cultured axenically, and toxin extracted from it behaved similarly to the shellfish toxin (331). A toxic substance was purified to the stage of a white amorphous solid (332, 333), but neither its chemical identity nor its exact relationship to the natural substance of the dinoflagellate have yet been clarified. The purified toxic substance with the molecular formula $C_{10}H_{17}O_4N_7$ has been hydrolyzed

to $C_8H_{10}ON_2$ to which was assigned the structure of a 2-oxopyrrolo(1,2-c) pyrimidine (334).

The poison from another dinoflagellate, *Gymnodinium veneficum*, obtained from nonaxenic cultures was shown to be toxic for many organisms including fish, amphibia, and mammals, although polychaetes were resistant (335). The authors pointed to the fact that extracts made with neutral alcohol were toxic, as were the cultures themselves, whereas acid extracts showed quite different pharmacological properties. The highly toxic venom of certain predaceous marine snails of the genus, *Conus*, has been considered to have as one of its actions interference with neuromuscular transmission (336). The nature of the toxins is not known, though paper chromatography of extracts of the venom apparatus showed the presence of N-methylpyridinium, homarine (N-methyl-2-picolinic acid betaine), and γ-butyrobetaine.

Tetramine (TMA), tetramethylammonium hydroxide, has been detected in extracts of several invertebrates. It was shown to be present in extracts of sea anemones (337). In addition, a curare-type paralysis for frog, fish, and mouse was shown to be given by tetramine. Welsh & Prock (338) detected spots on paper in the position of tetramine for extracts of six species of coelenterates; this was confirmed by Mathias, Ross & Schacter (193). The latter also noted that tetramine caused a contracture of frog rectus, an action which was antagonized by (+)-tubocurarine. These workers concluded that Richet's thalassine (339) could not be tetramine since the latter substance injected into dogs gave none of the thalassine effects. Tetramine was also found in extracts of the salivary glands of the poisonous mollusc, *Neptunea arthritica* (340, 341). Paper chromatography, infrared absorption, mixed melting point, and pharmacological behavior were used in identification. Originally, Fänge (342) and Emmelin & Fänge (343) believed that the salivary gland of *Neptunea antiqua* contained neurine (vinyltrimethylammonium hydroxide), but Fänge (344) later showed paper chromatograms in which spots of the extract agreed, not with neurine, but with tetramine. Welsh & Prock (338) speculated on the possibility that tetramine in nematocyst toxin might be responsible for the paralysis of crustacea stung by such organelles (345). It is questionable whether tetramine paralysis of crustaceans is of the simple myoneural type, however, since Cowan & Ing (346) found that this substance did not interfere with transmission in *Maia* nerve muscle preparations.

One almost untouched problem in invertebrate pharmacology relates to the action of neuromuscular blocking agents on invertebrates. The problems of the curarization of invertebrates and the actions of the curare-type substances are in such a poorly developed state that little is to be gained by reviewing the uncertain literature on this subject. Previous sections of this review have cited positive and negative effects of (+)-tubocurarine on invertebrate myoneural junctions. The possibility of neuromuscular blocking agents which are produced by invertebrates and act on invertebrates has been suggested but has been studied only slightly from a pharmacological

point of view. Once case (347) is that of the predaceous wasp, *Habrobracon juglandis*, in which the female stings its prey (larvae of *Ephestia*, *Plodia*, *Galleria*) causing paralysis, and then feeds upon the blood of the paralyzed larvae. The wasp larvae also feed upon the paralyzed prey, but the adult male wasps apparently have other food preferences, such as honey. Beard (347) considered the venom to produce a true neuromuscular block, and he presented some evidence in favor of this action. The venom is apparently very potent but its chemical nature is unknown, although Beard did find it to be water soluble, heat-labile, and nondialyzable. The paralyzing action is biologically specific and not all insects injected with the venom were paralyzed. This invertebrate counterpart of the South American Indian arrow poison would appear to be worthy of further study.

Pheromones

Following Karlson & Butenandt (30) we may define a pheromone as a substance secreted by an animal into its environment where it will act on another individual of the same species and excite that individual to specific activity. The examples which follow indicate the nature of some of the pheromones, the type of excitation elicited in the receiving individuals, and the biological role of these reactions.

The queen substance of the honeybee.—Through a series of careful observations and experiments it was possible to demonstrate that the honeybee maintains the integrity of its colony under the leadership of a single queen as the result of the effects of a substance produced by the mandibular glands of the queen. This queen substance, distributed over her body during grooming, is licked off by the workers and fed to other workers in regurgitated food (348). Direct contact with the queen, rather than sight, sound, or odor, seems to be necessary for inhibiting the development of the ovaries of the workers and thereby preventing their constructing another queen cell (349). The attractiveness of the queen for the workers was shown to depend on the presence of the queen's mandibular glands, for removal of these led to decrease in ability to attract workers (350).

Removal of the queen from the colony led to greater ovarian development in the workers than in the presence of the queen, but such development was suppressed by the presence of a dead queen (although an alcohol- or acetone-extracted dead queen did not possess this property). This inhibitory action was restored to the extracted dead queen's body if it was coated with the extract (351, 352). Additionally, an alcohol extract of the queen was able to suppress queen-cell production by worker bees (353). It was then shown that although haemolymph, brain, ovaries, and the worker's mandibular glands did not yield an extract capable of inhibiting queen-cell construction, the queen's mandibular glands did so (354). These glands were also shown to be the source of a substance capable of inhibiting development of worker's ovaries (355). Waxy crystals were obtained from an ethanol extract of mated queen's heads. The infrared spectrum indicated that the

queen substance was an α,β-unsaturated carboxylic acid containing another unconjugated carbonyl group (356). Complete reduction yielded a substance with a rate of flow in a gas chromatogram like that of decanoic acid: $CH_3(CH_2)_8COOH$. Catalytic hydrogenation yielded a ketonic acid identical with 9-oxodecanoic acid: $CH_3CO(CH_2)_7COOH$. The ultraviolet absorption of the dinitrophenylhydrazone, the infrared band at 1680 cm^{-1}, and the positive iodoform reaction led to the formulation of the queen substance as *trans*-9-oxo-2-decenoic acid: $CH_3CO(CH_2)_5CH{:}CHCOOH$ (357, 358). As related in a previous section, the queen substance is chemically closely related to the royal jelly acid, 10-hydroxy-2-decenoic acid. The biosynthetic origins and interrelationships of these acids are still unknown.

Sex pheromones.—A number of insects representing several orders utilize specific substances produced by the female which are liberated into the environment, attract the males of the same species (male "assembly"), and induce mating behavior and copulation (359). Valentine (360) described some simple but elegant experiments which demonstrated the presence of a sex attractant in the beetle (*Tenebrio molitor*). He showed that a fluid expressed from the tip of a female beetle's abdomen onto a glass rod would attract and excite the male to typical sexual behavior; a clean rod alone had no effect. A male whose elytra were rubbed with the female's secretion was also effective in eliciting sexual activity in another male, although normally males ignored other males and a glass rod contaminated with a male's secretions did not result in overt actions on the part of other males. The glass rod tipped with male secretion did attract females, but the resulting behavior was very variable and there were no indications of strong and persistent attraction. Valentine implicated the male's antennae as the recipient structures since an antenna-less male, though otherwise normal, or a male whose antennae were coated with paraffiin oil was not attracted in the typical manner to a female or to a glass rod tipped with the female secretions. Roth & Willis (361) reported that a beaker or a piece of filter paper with which female cockroaches (*P. americana*) had come into contact, was able, without the females, to excite males. Ether extracts of the contaminated filter papers showed the presence of a substance which also excited male cockroaches. Petroleum ether extracts of such filter papers were employed to demonstrate a quantitative relationship between the percentage of males responding and the concentration of the unknown substance (362). These sex pheromones are produced by glands in the posterior segments of the abdomen of the female (30, 363), but production of these substances is not constant throughout the life of the insect, and there is evidence that after mating there occurs a drop in the amount of attractant produced (364). The corpora allata, directly or indirectly, may be important in the production of the substance, for Barth (365) pointed out that removal of these glands from the Cuban cockroach reduced pheromone production as judged by reduction of "wing-raising display" of the males and failure to mate as evidenced by absence of spermatophores in the female's bursa copulatrix. Implantation of corpora

allata into allatectomized females showed evidence of restoration of pheromone production.

Recent developments have involved the successful chemical identification of two of these sex attractants. From the sacculi laterales of female moths of the silkworm (*Bombyx mori*) it was possible to obtain petroleum ether extracts which when deposited at the tip of a glass rod excited the male into vigorous wing flutter. This test was quantified (359) so that it could be employed in the assay of the active substance during the process of purification and isolation. In the initial experiments, begun in 1939, it was established that the active material was a neutral, nonsaponifiable alcohol with two conjugated double bonds. It was not until 1959 that the substance was isolated in sufficient amount for analysis and structure determination. From 500,000 glands was isolated the *p*-(*p*-nitrophenylazo)-benzoate of the active compound; this had a melting point of 95° to 96°C. and could be saponified to the free alcohol (366 to 369). The alcohol had very high activity; the response of the male moth was elicited by a concentration of 10^{-10} μg/ml. This activity means that only about 1000 molecules at the tip of the glass rod were necessary to give a response and that such a number would attract about 50 to 100 male moths. The actual number of molecules effective for one male moth must be very few indeed. Elementary analysis and ultraviolet absorption spectra indicated that the pheromone was a conjugated, doubly-unsaturated alcohol, $C_{16}H_{38}O$. Infrared spectra and identification of the products of catalytic hydrogenation and of oxidative degradation of the alcohol led to the structure $CH_3(CH_2)_2CH{:}CHCH{:}CH(CH_2)_8CH_2OH$; 10,12-hexadecadien-1-ol, or bombykol. Synthesis of the four possible geometric isomers of this doubly unsaturated alcohol (370) revealed that the physical properties of bombykol did not agree with those of either the 10 *cis*, 12-*cis* isomer or the 10-*trans*, 12-*trans* isomer. The biological activity of the four isomers in terms of the quantity per sex-attractant unit was: 10-*cis*, 12-*cis*-hexadecadienol (1 μg/ml); 10-*trans*, 12-*trans*-hexadecadienol (10 μg/ml); 10-*cis*, 12-*trans*-hexadecadienol (10^{-3} μg/ml); 10-*trans*, 12-*cis*-hexadecadienol (10^{-12} μg/ml). Bombykol had an activity of 10^{-10} μg/ml. It was therefore concluded that bombykol is the 10-*trans*, 12-*cis* isomer. The second sex pheromone whose chemical constitution is known is that of the female gypsy moth (*Porthetria dispar*). The structure was established after 30 years of work at the United States Department of Agriculture (371). The substance, obtained from 500,000 virgin females, was identified as (+)−10-acetoxy-1-hydroxy-*cis*-7-hexadecene:

$$CH_3(CH_2)_5\underset{\displaystyle OCOCH_3}{\underset{|}{C}}HCH_2CH{=}CH(CH_2)_5CH_2OH$$

The racemic form was synthesized and was shown to have about the same activity for male moths as the natural substance. These two discoveries are events of major scientific importance, not only because of the practical possibilities of their application to insect control but also because these

extremely active and specific substances offer unique possibilities in cellular physiology and pharmacology of sense organs. The stimulation of the male antennal receptors by the female sex attractant was demonstrated by recording the slow changes in potential and the summed discharges evoked in a male antenna following exposure to an extract of the female abdominal glands (372 to 374). The female antenna did not respond to the female attractant, but there were no differences on the part of the male and female in their responses to an artificial substance such as sorbinol and cycloheptanone. Studies of the antennal receptors by the four geometric isomers of 10,12-hexadecadien-1-ol should offer a unique opportunity to examine by electrophysiological methods a sensory system of very great specificity and sensitivity.

Polypeptides and Proteins

Invertebrates, like vertebrates, employ various large molecular weight substances for specific functions of a pharmacodynamic nature. Enzymes, toxic proteins, or polypeptides are known or suspected to occur in bee venom (5, 25), wasp venom (25, 287), *Physalia* nematocysts (375), coelenterates (193, 376), tiger moth (327), octopus saliva (283), and assassin bug saliva (377). A substance of more than passing interest is hirudin, the antithrombin of the leech. A considerable purification of hirudin by paper chromatography and electrophoresis was recently achieved (378 to 380). It was considered to be a polypeptide with a molecular weight of about 16,000. The amino acids were determined and an isoelectric point of 4.7 was assigned to hirudin (380). It was suggested that hirudin rapidly complexes with thrombin, perhaps molecule per molecule. The free-COOH groups (but not the free-NH_2 groups) of hirudin were thought to be important in the formation of this complex, as were the free-NH_2 groups of thrombin. An inhibitor of blood clotting (tabanin) from the salivary glands of the blood-sucking Tabanid flies was recently described (381, 382). It did not react with prothrombin or fibrinogen but did appear to form a stoichiometric complex with thrombin. Tabanin, too, may be a polypeptide.

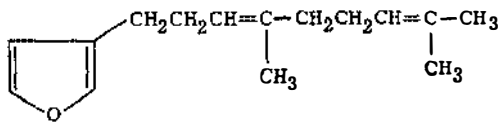
Special Substances Produced by Terrestrial Invertebrates

Many insects and other terrestrial invertebrates produce substances useful in offense, in defense, and in certain other activities. These substances are of diverse nature varying from simple compounds such as formic and oxalic acids to more complex organic molecules such as terpenoid and sesquiterpenoid compounds. In addition to the interesting and still unsolved problems concerning the origin and biosynthesis of these substances in the organisms producing them and the mechanisms of action on prey or enemy organism, there is the possibility that some of these unique substances may offer new or additional means to aid the pharmacologist along theoretical, or practical lines, or both.

Certain insects possess scent glands that produce odoriferous or pungent

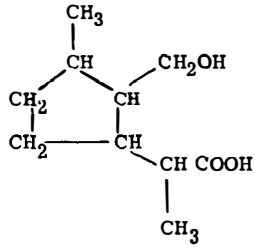
substances which have a repellent function or serve for the laying down of identifying trails or markers (30). Certain ants (Formicinae) are known to produce relatively large quantities of formic acid (383, 384) which, used as a spray, can act in offense or defense. The mechanism of formation of this acid and the ant's means of protecting itself against concentrations as high as 20 to 70 per cent formic acid are unknown. The same questions exist with respect to certain Dipterous larvae of the genera *Platyura* and *Ceroplatus*, which apparently produce oxalic acid and which are able to withstand contact with 0.15 per cent oxalic acid (385). The Formicine ant *Dendrolasius fuliginosus* produces, in addition to formic acid, a substance with a strong odor like that of citronella (26). This substance, emitted when the ant is irritated, is produced in the mandibular glands.

A petroleum ether extract of the ants was made, and purification by fractional distillation yielded a substance called dendrolasin which proved to have the following structure:



Dendrolasin is an isoprenoid compound chemically related to certain plant substances, i.e., α-clausenane and perillene. Dendrolasin was found to be a selective contact insecticide acting rather specifically on ants and having little or no action on certain beetles, bugs, orthopterans, and other insects. It was also ineffective toward workers of the species producing the substance (26). In another ant (*Chthonolasius umbratus*) two nonisoprenoid substances, unecane ($C_{11}H_{24}$) and methyl-*n*-undecyl ketone ($CH_3COC_{11}H_{23}$), have been found.

The Dolichoderinae ants do not produce formic acid but have anal glands which secrete a number of physiologically active substances. The Argentine ant, *Iridomyrmex humilis*, was found to have a monoterpenoid lactone, iridomyrmecin (melting point 59° to 60°C., $[\alpha]_D^{17}+205$°C.) (386). Ethyl ether extraction and purification by sublimation finally gave the pure crystalline iridomyrmecin (386). Elucidation of the structure showed iridomyrmecin to be one of the stereoisomeric forms of the lactone of α-(2-hydroxymethyl-3-methylcyclopentyl)-propionic acid (387) (see also Fig. 1):



The structure elucidation was facilitated by the finding that iridomyrmecin, upon oxidation, yielded a di-acid identical in melting point (117 to 118°C.) with one of the nepetalinic acids derived from nepetalactone, a substance in oil of catnip (*Nepeta cataria*), an aromatic plant in the family Labiatae. All of the evidence indicated that iridomyrmecin was present only in the anal gland secretion of the workers and queens. Iridomyrmecin was found to have only weak antibacterial activity and no exceptional pharmacological or toxicological actions on mammals. It is a good contact insecticide, killing even the species of ant producing it. In addition it was capable of inhibiting growth of seedlings of *Lupinus albus* (386).

Meanwhile, work in Australia not only confirmed the presence of iridomyrmecin in the Argentine ant, but also demonstrated the presence of other terpenoid compounds in various Dolichoderine ants. From *I. nitidus* was extracted an iridolactone (mp 58 to 59°, $[\alpha]_D^{17}$-62°C.) (388, 389) with the same formula ($C_{10}H_{16}O_2$) as iridomyrmecin but not identical to it. Oxidation of this new iridolactone yielded a di-acid (mp 81 to 82°C.) which was identical with the nepetalinic acid epimeric at C_8 with the nepetalinic acid (mp 117 to 118°C.) derived from the oxidation of iridomyrmecin. The new iridolactone was therefore recognized as *iso*iridomyrmecin (Fig. 1), earlier obtained (387) by epimerization of iridomyrmecin. The presence of a terpenoid dialdehyde called iridodial was demonstrated in *Tapinoma nigerrimum* (26), in *I. detectus*, a meat ant, and in *I. conifer*, the stick ant (388, 390). This dialdehyde was shown to have the structure given in Figure 1. Still another dialdehyde, dolichodial, was found in *Dolichoderus acanthoclinea* (391). Pavan (25) observed that iridodial has no odor and no insecticidal action.

Other substances, including methylheptenone and *n*-propyl *iso*butyl ketone, were found in the three ants which secrete iridodial (25, 388, 392). The anal secretion of *Tapinoma nigerrimum* contains methylheptenone, *n*-propyl *iso*butyl ketone and iridodial. The first two of these are responsible for the characteristic odor of the insects and for the insecticidal action of the poison. Pavan has expressed the view that the function of the relatively nonvolatile iridodial is to prevent the evaporation of the two more volatile ketones and thus to prolong the insecticidal effectiveness of the material when used as a defensive measure.

There appears to be only a limited structural specificity for insecticidal action in these lactones. Natural iridomyrmecin and D(+)- and L(-) *iso*iridomyrmecin all have about the same insecticidal activity (393). Cavill and his co-workers (394) have prepared a number of synthetic 1,5 dialdehydes and the related cyclopentano-valerolactones and found that insecticidal activity is absent in the synthetic compounds that lack the carbon skeleton of the natural iridolactones. Further exploration of the relationship between structure and insecticidal activity in these lactones should provide interesting information.

It is of interest, though not of pharmacological relevance, to note that, besides nepetalactone, compounds with the carbon skeleton of the iridolactones are becoming recognized as of rather frequent occurrence in nature.

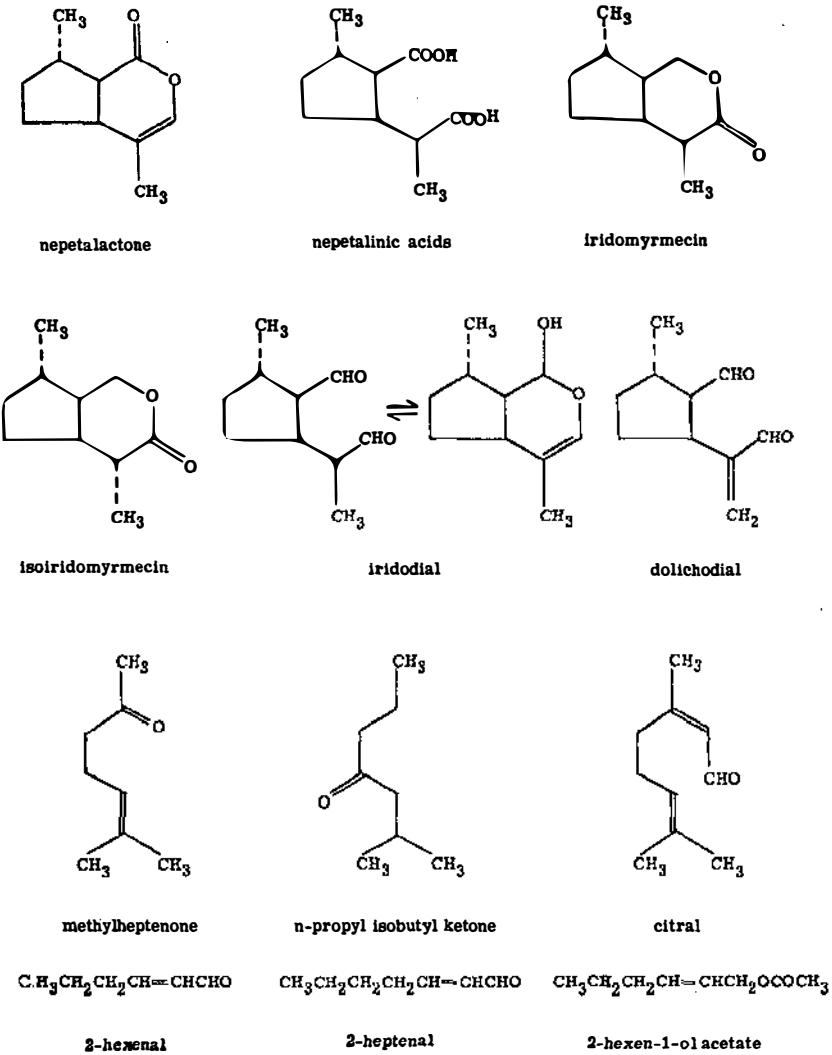


Fig. 1. Compounds occurring in Dolichoderine ant extractives and in other insects.

A number of plant substances possess the same arrangement of carbon atoms and are cyclopentanopyran derivatives. Among others, these include verbenalin (395), aucubin (396), and genepin (397). Matatabilactone and actinidine, the latter a related alkaloid, are of special interest; the former is an iridolactone or a dihydronepetalactone, and both the lactone and the alkaloid are highly attractive to cats and related species (398).

Acyclic compounds.—*Trans*-2-hexenal is found in insects of several orders. It has been isolated from one West African black cocktail ant (*Atpogyne*

africana) (399), the cockroach (*Eurycotis floridana*) (400), and the bug, *Acanthocephala femorata* (401). The related 2-heptenal is found (along with tridecane) in the rice stink bug, *Oebalus pugnax* (402), and 2-hexen-1-ol acetate has been obtained from the male water bug, *Belostoma indica* (*Lethocerus indicus*) (403). In addition to these closely related, nonterpenoid compounds, citral (see Fig. 1) occurs in the leaf-cutter ant, *Atta sexdens rubropilosa* (404). The occurrence of citral in an ant is of special interest in view of the suggested role of this terpenoid aldehyde in the biosynthesis of the iridolactones (393). The presence of 2-hexenal and 3-hexenal in mulberry leaves is said to be responsible for their attractiveness to silkworm larvae (405).

The exact physiological significance of these secretions is still not known with certainty. They may act as sex attractants or excitants (403), or as insect repellents in defense against attack (400). The bug, *Acantocephala femorata*, ejects 2-hexenal when approached by fire ants (*Solenopsis saevissima* var. *richteri*) and ejects it in copious amounts when under attack. *Oebalus pupnax* uses its secretion, containing 2-heptenal, in the same way.

Quinones.—These chemically reactive compounds occur in both plants and animals and have diverse functions (13). The possible role of *o*-quinones in the tanning reaction has already been indicated (249). A number of *p*-quinones have been found in insects in which they appear to play a role in defensive and repellent behavior and possibly other functions (26). Since the publication of evidence (406) indicating that ethyl-*p*-benzoquinone occurs in the flour beetle (*Tribolium castaneum*), a number of reports appeared showing that *p*-quinones occur in several orders of insects. Ethyl-, methyl-, and methoxy-*p*-benzoquinone were reported to occur in *T. castaneum*, the first of these occurring as the principal quinone (80 to 90 per cent of the total) and the last being found only in traces (407). These were found to be present in males and females in both abdominal and thoracic glands of adult beetles, but apparently neither larvae nor prepupae produce these quinones. They appeared at the thorax only in older pupae or soon after emergence of the adults. The abdominal secretions appeared later after emergence (408). The tenebrionid beetle, *Diaperis maculata*, was also found to produce ethyl- and methyl-*p*-benzoquinone in a pair of abdominal glands and a pair of thoracic glands (409). *p*-Benzoquinone, ethyl-, and methyl-*p*-benzoquinone were found in the cockroach (*Diploptera punctata*) in male and female roaches and in the nymphs as well as the adults (409). Such quinones apparently occur also in myriapods (409).

The irritating quality of this secretion and its role in defense are illustrated by an experiment with one of the bombardier beetles (410). Beetles of the genus *Brachynus* are able, when attacked, to repeatedly eject from the tip of their abdomens a fine spray. Eisner demonstrated that one of these beetles was able to direct the spray correctly in the direction of the attacker and the ejected material repelled an ant, a carabid beetle, a mantid, and a spider. These animals were not killed but showed temporary signs of incapac-

itation. The material of 30 expulsions was collected from one of these beetles (*Brachynus crepitans*), and *p*-benzoquinone and methyl-*p*-benzoquinone were identified (411, 411a). The false wireworm *Eleodes hispilabris* elevates its abdomen and ejects accurately at an attacker a pungent oily spray in which ethyl- and methyl-*p*-benzoquinone are present (412). Attacks by imported fire ants were successfully repelled by the beetle, the ants retreating after being sprayed and showing signs of motor incoordination. The beetle's body was covered by its own spray but it was apparently unharmed by the secretion. It has been shown that in some beetles (*T. confusum*) exposure to their volatile secretions leads to toxicity or the production of abnormalities (408). There are also examples of organisms which are quite resistant to these gaseous quinones. *T. confusum* is often heavily infested with mites (*Acarophenax tribolii*) which are apparently not affected adversely by the quinones.

Spiders of the family Gonyleptidae give off from glands in the cephalothorax a volatile antibiotic which was found to be effective against both protozoa and bacteria (413). This gonyleptidine was later found to have 2,3-dimethyl-1,4-benzoquinone as a major constituent and 2,5-dimethyl-1,4 benzoquinone and 2,3,5-trimethyl-1,4-benzoquinone as minor constituents (414). A number of *p*-quinones, both naturally occurring and synthetic, are known to be bacteriostatic (415), one theory of this action being the destruction of free-sulfhydryl groups by addition to the conjugated system.

Pederin.—It has been known for some time that beetles of the genus *Paederus* are noxious to man and animals, contact with these beetles causing cutaneous or ophthalmic lesions, or both. A crystalline substance, pederin, whose chemical nature remains undetermined, was obtained from *Paederus fuscipes* (416). It was shown to be very potent in mice in producing cutanecus necrosis, loss of hair, and severe dermatitis. The biological and chemical properties of pederin indicate that it is not identical with cantharidin, the well-known vesicant of certain beetles whose chemistry, pharmacology, and toxicology are nicely summarized by Kaiser & Michl (5).

The wide diversity of chemical structures found in the venomous, poisonous, odorous, and other pharmacologically and physiologically active substances produced by invertebrates and the relatively small portion of the enormous world population of these animals so far examined suggest that large numbers of pharmacologically interesting substances still await discovery. We hope this review will serve to stimulate the interest that will lead to further explorations in this field.

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