CHEMICAL PROPERTIES AND PHYSIOLOGICAL ACTIVITY OF A NEUROACTIVE COMPONENT FROM THE VENOM OF *CONUS CALIFORNICUS*

Thesis by

Ellen Elliott

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

(Submitted March 19, 1975)

ACKNOWLEDGMENTS

I feel that in the completion of this project I have done little more than coordinate the efforts of a lot of experts in a variety of fields. I would like to thank all those experts, beginning with JacSue Kehoe, a masterful neurophysiologist and very generous person, who took me into her home as well as her lab and contributed immensely both to the substance of my work and to my attitude toward it. I am also very grateful to Ken Reed who, confronted with the combination of little sample and much water, spent many hours obtaining for me an NMR spectrum of conusine, after much singing of the water peak blues. I would like to thank Professor Donald Jenden who very generously devoted much time working to obtain a mass spectrum of conusine. I appreciate the help of Juta Reed, an expert biochemist, who ran the ATPase assays on conusine and served as my biochemistry consultant. I would like to thank collectively the numerous individuals at Caltech who have helped me in many ways--from photographing my Conus to helping with innumerable bioassays which have not been included in this thesis. Also, my thanks goes to Dick Vandlen, for supplying acetylcholinesterase, α-bungarotoxin and miscellaneous comparative toxicology information. I am very grateful to my advisor, Mike Raftery, for his indulgence in allowing me the freedom to pursue my maverick project as it serendipitously developed. Most especially of all, I thank Bob for his tremendous patience and supportiveness, both professional and personal, in bearing my anxieties and misgivings throughout my research and writing, and for his criticisms and contributions to this work.

Fortunately, I have benefited from the help of as many funding agencies to support me as experts to work with me. I would like to acknowledge the National Science Foundation, Calbiochem, and the National Defense and Education Act for fellowships and the Biology Division of Caltech and my advisor Mike Raftery for additional financial assistance during the various years of my graduate research at Caltech.

ABSTRACT

A small molecular weight component, named conusine, was purified from the venom of the marine snail Conus californicus. The solubility properties, enzyme susceptibilities, heat and pH sensitivity, spectral characteristics and reactivity with various indicator reagents of conusine were determined. Conusine was found to inhibit the excised, spontaneously beating heart of the clam Mercenaria mercenaria. This inhibition was blocked by those antagonists which were found to block acetylcholine inhibition of the heart. In addition, in the medial cells of the Aplysia pleural ganglion iontophoretically applied conusine was found to activate selectively a cholinergic receptor which mediates a slow hyperpolarization of the cell due to an increase in K^+ permeability. Conusine was concluded to be a cholinergic agonist specific for this type of slow hyperpolarizing receptor. From additional pharmacological studies of various cholinergic agonists and antagonists on the clam heart, this tissue was hypothesized to contain the same type of slow hyperpolarizing cholinergic receptor as the medial cells of the Aplysia pleural ganglion.

ABBREVIATIONS USED

ACh	acetylcholine
AChE	acetylcholinesterase
ASW	artificial sea water
Bgt	α-bungarotoxin
BTX	batrachotoxin
BuChE	butyrylcholinesterase
BZQ	benzoquinonium (WIN 2747, Mytolon)
CNS	central nervous system
DPA	dipicrylamine (2, 2', 4, 4', 6, 6'-hexanitrodiphenylamine)
dTC	d-tubocurarine
DTT	dithiothreitol (Cleland's reagent)
5-HT	5-hydroxytryptamine (serotonin)
MXC	methylxylocholine (β-TM 10)
NTX	nereistoxin
PTMA	phenyltrimethylammonium
SW	sea water
TEA	tetraethylammonium
TLC	thin layer chromatography, thin layer chromatogram
TLE	thin layer electrophoresis
TMA	tetramethylammonium (tetramine)
TTX	tetrodotoxin

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

INTRODUCTION

The work described in this thesis includes the purification of a component from the venom of the California cone snail and its assay on a number of biological preparations. To provide background for this work, the following introduction presents a brief description of cone snails and the research which has been done on their venoms, and an overview of the relevant background material concerning the two major preparations investigated.

1. Cone Snail Venoms

Naturally occurring toxins from both plants and animals have proved to be invaluable tools in the study of many aspects of animal physiology, most notably in the study of neurophysiological function (see 80, for review). The usefulness of neuro-active toxins from such sources arises from their great specificity for particular components of the nervous system. Two of the best known examples, for instance, are tetrodotoxin (TTX), which serves as a very specific probe for the voltage-dependent Na^+ ionophore involved in nervous conduction (78), and α-bungarotoxin (Bgt), which serves as a label for the acetylcholine (ACh) receptor involved in neuromuscular transmission (70). These two toxins have been used extensively and profitably in the study and characterization of their respective target molecules (42, 92).

Marine organisms offer an abundance of venoms and toxins, relatively few of which have been isolated and biochemically identified

(88, 89). The marine snail family Conidae, containing the single genus Conus with about four hundred known species (37), has long been known for its venomous members, several of which have been responsible for human injuries and even fatalities (67, 76). The majority of these species are found in tropical and subtropical regions of the world. A single species, Conus californicus Hinds, is found off the coast of North America from northern California to the southern tip of Baja California (38).

Cone snails are predacious and possess an elaborate venom apparatus, including a highly coiled venom duct which secretes the venom, a muscular bulb which presumably aids in propulsion of the venom along the duct (there is disagreement as to whether this bulb also produces venom; 25, 68, 76), a radular tooth modified for "harpooning" and envenomation of the prey, and a radular sheath which houses the store of radular teeth (14, 40, 43; see Figure 1). The venom duct opens into the pharynx just posterior to the point where the radular sac joins the pharynx. The hollow, chitinous radular teeth, formed in the long arm of the radular sac and stored in the short arm, are released one at a time into the pharynx where they are charged with venom and then moved to the anterior end of the proboscis (37; see Figure 2). The prey is impaled with this venomous tooth, which is grasped by the muscular tip of the proboscis and which may be retained by the proboscis after injection and used to pull in the prey (65) or which may be released after injection into the prey, depending on the species of Conus (76). Envenomation generally results in paralysis of the prey.

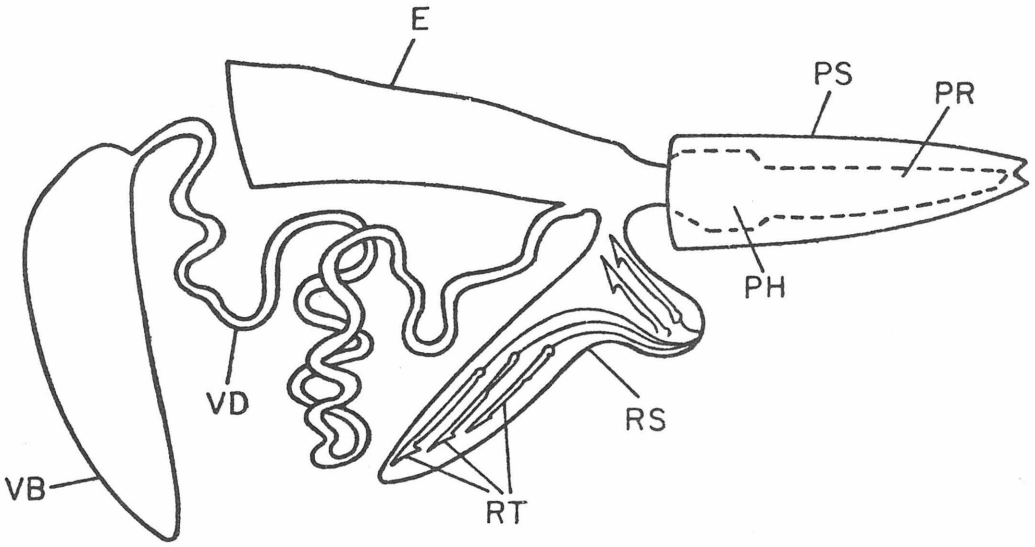


Figure 1. Venom apparatus of Conus. E, esophagus; PH, pharynx; PR, proboscis (retracted); PS, proboscis sheath; RS, radular sac; RT, radular teeth; VB, venom bulb; VD, venom duct (after 68, 76, 88).

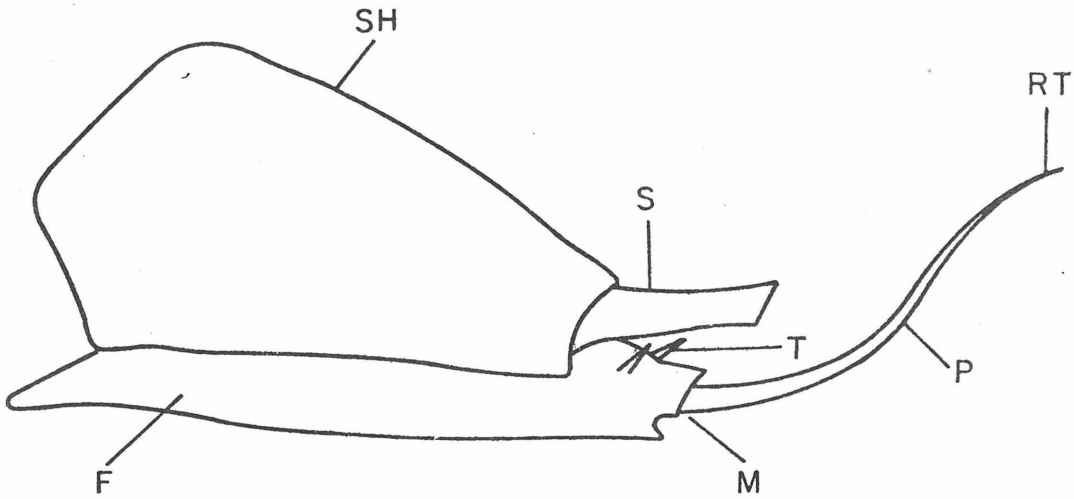


Figure 2. External features of Conus. F, foot; M, mouth; P, proboscis; RT, radular tooth; SH, shell; S, siphon; T, tentacle (with eye).

The nature of the prey varies with the species of Conus. In a comprehensive study of Hawaiian Conus species, Kohn has reported that a given species shows a relatively high degree of selectivity in its diet, some cones feeding exclusively on gastropods, others on fish, and others on polychaete worms (66). C. californicus does not show this selectivity, having been observed to eat a great variety of gastropods, pelecypods, polychaetes, fish, and even an octopus and a tectibranch (91). However, only in the case of gastropods and polychaetes did the cone snail actively prey on its food with stinging preceding feeding, indicating that C. californicus will feed on a variety of dead organisms and suggesting that its venom need not be potent against all of them. Saunders and Wolfson (91) have described the paralysis of gastropods after C. californicus stinging in aquaria but reported that polychaete worms continued to writhe after stinging. In the course of the studies described in this thesis, C. californicus were often observed to sting and eat polychaetes and earthworms, which reacted violently to the sting immediately afterwards, but which showed a gradual decrease in and often cessation of activity with time.

Endean and Rudkin (25, 26) have reported that, for a number of Australo-asian species of Conus, both the toxicity of the venom and the morphology of the radular teeth of a given cone species appear to be related to the natural prey of that species. In experiments involving injection of whole venom into selected test organisms, they found that only the venoms of piscivorous cones were toxic to vertebrates (mice and fish) and that such venoms were without effect on molluscs and

polychaetes. In turn, the venoms of molluscivorous and vermivorous cones were without much effect on mice and fish (except, occasionally, for necrosis) and were, in general, toxic though not always paralytic to the gastropod and/or polychaete test species. Toxicity in mice was generally manifested as flaccid paralysis (except in the case of C. magus venom, which caused spastic paralysis; see below) and respiratory failure leading to death, with maintenance of cardiac function for several minutes after respiratory arrest. Fish showed blanching, flaccid paralysis (except, again, in the case of C. magus) and eventual respiratory failure. Toxic symptoms in gastropods were, similarly, apparent flaccid paralysis and death, with the heart continuing to beat for as long as 24-48 hours after venom injection.

Somewhat in contrast to this general rule that the venom is specific for the prey, Whysner and Saunders (113) have shown that C. californicus venom is toxic to vertebrates. They reported that intravenous injection of the whole venom into mice caused ataxia, inactivity alternating with hyperexcitability, loss of righting reflex, convulsions, and death by respiratory arrest. In cannulated rabbits, the injected whole venom caused hypotension, transient bradycardia, and progressive respiratory failure leading to death, with no cardiac arrest until after respiratory failure. Although artificial respiration restored the heart beat temporarily, it did not prevent death.

The above studies indicate that the findings on the venom of one species cannot necessarily be extrapolated to the venoms of other species. Even within such groupings as piscivorous, molluscivorous,

or vermivorous cones, there are differences in toxic effects of the venoms (25, 26), indicating different active principles.

Reports of Conus stings in man indicated that the resultant symptoms fall into two categories--mild and severe (76). The symptoms of a "mild" reaction are primarily local and include sharp pain, sometimes numbness, and usually redness at the site of the bite, with occasional swelling, edema and headache. The "severe" symptoms include sharp stinging pain and numbness at the site of the bite, followed by difficulty in swallowing and speaking, respiratory distress, excessive perspiration, lacrimation and salivation, tremor, and with flaccid paralysis eventually spreading to all parts of the body (76, 88). Death is usually by respiratory arrest, though in some cases death is preceded by a coma and has been prescribed as due to cardiac failure (37). In those cases in which identification of the species responsible for human stinging is certain or fairly certain, it appears that piscivorous cones, in particular, C. geographus, are responsible for most of the severe symptoms and all the fatalities, while molluscivorous or vermivorous cones generally inflict only mild stings (26, 76). Thus, there appears to be a toxicological basis for the manifestation of two distinct symptomologies of stinging in humans. In the cases of C. californicus stinging of man which have been reported, the resultant symptoms are mainly local erythema and edema (F. E. Russell, personal communication). No reports of human fatalities due to C. californicus stings are known (113).

Several groups of investigators have studied the effects of Conus venom on isolated organ preparations and attempted to determine the site of toxic activity more definitively. Endean and coworkers have looked at the effect of whole venoms of several piscivorous species of Conus on the isolated phrenic nerve-diaphragm of the rat (23, 24, 115) In their experiments the diaphragm and phrenic nerve were stimulated with extracellular electrodes and the muscle tension of the diaphragm was recorded with an ink-writing gimbal lever. In the case of C. geographus venom, which produced flaccid paralysis when injected into mice, Whyte and Endean (115) found that the venom reversibly blocked contraction of the diaphragm in response to both direct electrical stimulation of the muscle and "indirect" nervous stimulation. Endean and Izatt (23) found that the venom of C. magus, which they observed to cause spastic paralysis in mice and fish, had a dual effect on the phrenic nerve-diaphragm preparation. In addition to blocking muscle twitch in response to direct and indirect stimulation, the venom produced a marked baseline contraction of the muscle cells. The acetylcholinesterase(AChE) inhibitors eserine and neostigmine, d-tubocurarine (dTC, which blocks the depolarizing action of ACh at the neuromuscular junction), and ACh itself all had no effect on either aspect of the venom activity, which was slowly reversible with extensive washing. Finally, Endean, Izatt, and McColm (24) reported that the venom of C. striatus from about half the specimens used (7 out of 11) elicited both of the responses observed with C. magus venom, i.e., blockade of muscle twitches along with a very slight baseline contraction of the muscle.

The venom of the remainder of the specimens elicited only the response seen with C. geographus venom, i.e., blockade of muscle twitch. The investigators suggested that the two actions of C. magus venom were due to two different components, one of which was present to varying degrees in the venom of C. striatus.

Freeman and Turner have further investigated the effects of fractionated venom from C. magus and that from C. achatinus, a species with a similar venom, on intact rats and rabbits as well as on a variety of isolated preparations, including the rat phrenic nerve-diaphragm, rat ileum, toad sciatic nerve-sartorius muscle, and the isolated hearts of guinea pig and rabbit (31). From the effects of Conus toxin on these systems, they concluded that the toxin was not impeding nerve conduction nor causing heart failure (though it did cause bradycardia and effect a number of changes in the cardiac action potential).

The preparation which Freeman and Turner studied in greatest depth was the rat diaphragm, from which they recorded intracellularly in addition to measuring muscle tension. They confirmed the observations of Endean and co-workers that C. magus venom, and additionally that of C. achatinus, caused a depolarization of the muscle (from an average of 73 mV to 41 mV) and a blockade of muscle twitch in response to both direct and indirect stimulation. Furthermore, they found that TTX, low (5 mM) external Na^+, and procaine (which depresses membrane permeability to cations in general) all caused essentially complete reversal of Conus toxin-induced depolarization of the muscle (31,101). That is, reducing the external Na^+ or adding TTX or procaine to a

diaphragm depolarized by Conus toxin caused repolarization of the muscle. Upon washing, the muscle membrane depolarized again, indicating that the toxin was still bound. Only after extensive and prolonged washing was the normal membrane potential restored and even then, never to quite the original value. These results are similar to those observed by Albuquerque et al. (2) for batrachotoxin (BTX), which depolarizes both nerve and muscle cells by irreversibly increasing membrane permeability to Na^+. As Freeman and Turner note, however, the action of Conus toxin differs in several respects from that of BTX (31), e.g., Conus toxin appears to be much more reversible than BTX and does not show the pronounced effects on cardiac muscle which BTX shows. A number of other effects of the Conus toxin on the various preparations which Freeman and Turner investigated suggested the involvement of Ca^{++} or changes in Ca^{++} binding. Thus these investigators have hypothesized that either Conus toxin acts, in the manner of BTX, directly to increase Na^+ permeability of membrane with resultant changes in Ca^{++} distribution, or Conus toxin acts primarily by altering membrane permeability to Ca^{++}, with depolarization due to Na^+ influx as a secondary effect (101).

Finally, in a brief note regarding some preliminary experiments, Cottrell and Twarog reported the effect of a venom extract from C. californicus on the buccal ganglion of the snail Helix aspersa (18). [In choosing this assay system, Cottrell was evidently extrapolating his results from another Helix species, Helix pomatia, in which he has described the innervation of three giant cells in the buccal ganglion

by each of two giant cells in the cerebral ganglion, via the cerebro-buccal connectives (16). This synapse may well be serotonergic, since the giant cerebral ganglion cells have been shown to contain serotonin (5-hydroxytryptamine, 5-HT; 17), bath application of 5-HT depolarizes the buccal ganglion cells, and the 5-HT antagonists lysergic acid diethylamide and methysergide block the response of the buccal cells to stimulation of the cerebro-buccal connectives (15).] Cottrell and Twarog reported that the whole venom from C. californicus caused both depolarization and spike firing of the buccal ganglion cells, as well as blockage of their response to stimulation of the cerebro-buccal connectives. From these results, they concluded the presence of 5-HT in the venom as well as a factor which blocked synaptic transmission at this serotonergic synapse and a factor which blocked axonal conduction (18). The evidence was not detailed, however, and it is not possible to determine the basis for, nor to evaluate, all of their conclusions.

The above survey of investigations on the physiological effects of cone venoms on whole organisms and on isolated organ preparations demonstrates that the research efforts on this problem are at an early stage. Purification of the toxins immediately suggests itself as a next step in the study of their toxic effects. Animal venoms generally contain a plethora of physiologically active components, and the effect of the unfractionated venom represents a variety of responses. Thus purification and isolation of single active components is necessary before the exact site of their effect can be reliably determined.

The only isolation step used so far in the purification of Conus venoms has been gel filtration on molecular sieving columns. Cottrell and Twarog (18) performed a Biogel P-2 fractionation of C. californicus venom and found the agents active on the snail ganglion system to elute between the void volume and total column volume, i.e., somewhere in the molecular weight region of 800-1800 daltons. Whysner and Saunders (114) fractionated C. californicus venom on a Sephadex G-200 column and located the component lethal to mice in about six consecutive fractions, but no calibration curve or elution volumes were given, so that it is impossible to derive a molecular weight estimate for the lethal component. Freeman and Turner (31, 101) in their experiments fractionated the C. magus and C. achatinus venoms on a calibrated Sephadex G-50 column and found the toxin which affected rat diaphragm to elute in the region of 10, 000 molecular weight.

Attempts to characterize biochemically the toxic principles of cone venom have been preliminary as well. Kohn et al. (68) reported that both C. textile and C. striatus venoms were stable to heating at 100°C for 5-10 min, that trypsin incubation did not destroy the activity of C. striatus venom (though in general it increased the time required for action, i.e., the fish on which the venom was assayed died about 20-30 min later, on the average), and that the activity of C. striatus venom was not lost upon dialysis. Whysner and Saunders (114) have reported that the toxic principle of C. californicus venom(as assayed by injection into mice) was not stable to heating at 100°C for 5 min, was precipitated in distilled water, was non-dialyzable, and was

destroyed by pH 3.1-2.6 and partially attenuated by pHs of 11.0-10.7 and 4.5-5.2. Neither of these reports conclusively show the respective toxins to be either protein or nonprotein. Freeman and Turner found the toxins from C. magus and C. achatinus venoms to be stable to heating at 100°C for 10 minutes at pH 6 or 7.2 but not at pH 8.5. They also reported that the toxin did not absorb significantly at 280 nm (31).

Endean, Izatt, and McColm (24) described the presence, in the venom of C. striatus, of elipsoid granules from $3.5\mu \times 2.5\mu$ to $9\mu \times 4\mu$ in size. By histochemical staining of these granules, they concluded the presence of a thin polysaccharide film on the outside of the granule, a proteolipid sheath, and a core containing protein and 3-indolyl derivatives. The relationship of these granules to toxic activity was not investigated. Kohn et al. reported, also in the venom of C. striatus, the presence of the quaternary ammonium compounds homarine (N-methylpicolinic acid), γ-butyrobetaine, and N-methylpyridinium. None of these compounds was considered to be responsible for the toxic effects of the venom (68; J. H. Welsh, personal communication).

It is clear from the experiments which have been done to date on Conus venoms that these venoms contain some potent toxins. The purification and further study of these toxins is sure to deepen our appreciation for the diversity of venomous mechanisms which have developed, and may additionally supply new molecular tools for the study of physiological processes.

2. Cholinergic Action in the Clam Heart

In the experiments in this thesis, the heart of the clam Mercenaria mercenaria (L.) (formerly Venus mercenaria Linnaeus) was originally chosen as a quick, simple bioassay in which to screen Conus venom for possible serotonergic effects. The Mercenaria heart is excited by very low concentrations of 5-HT, which is probably the transmitter of the excitatory regulatory nerves (73). Since, however, Conus venom was found to have a cholinergic-like effect on the clam heart, it is the history of the investigation of cholinergic action in this system which is relevant and which is reviewed below.

The inhibitory (negative inotropic and chronotropic) effect of ACh on clam hearts has long been known (69). Prosser (84) first quantitated the extraordinary sensitivity of excised, spontaneously beating Mercenaria hearts to ACh applied to the bath. He reported thresholds of activity as low as 10^{-12} g/ml ($\sim 5 \times 10^{-12}$ M). Soon after, Wait (105) determined the dose-response curve of ACh action on the heart, and reported a comparatively narrow range between threshold concentrations, $\sim 5 \times 10^{-12}$ g/ml, and saturation concentrations which caused complete diastolic arrest, 5×10^{-10} g/ml. The Mercenaria heart was first used for bioassaying ACh by Welsh (108), who described it as a quicker, more sensitive and less delicate preparation than the previously used ACh bioassay systems of leech dorsal muscle, frog rectus abdominis, and frog heart. Subsequently, the clam heart became a standard bioassay system, widely used for the routine detection and

estimation of ACh (29) until more specific chemical methods were developed.

Accumulated results from many investigators indicate that ACh is almost certainly the natural transmitter of the inhibitory cardio-regulatory nerves in Mercenaria heart. Jullien et al. (54) reported the presence of ACh in the heart tissue and Welsh and Taub (110) measured the concentration to be ~0.5-0.7 μg ACh/g tissue (compared to 5 μg/g in Mercenaria nerve ganglia and 0.1 μg/g in adductor muscle). A number of investigators (54, 87, 94) have detected the presence of AChE activity in Mercenaria heart, although in very low amounts. Electrical stimulation of the visceral ganglion (from which the cardio-regulatory nerves arise) produces an inhibition of the heart indistinguishable from that produced by exogenously applied ACh (84). Eserine and neostigmine prolong the inhibition due to stimulation as well as that due to applied ACh (84,105,107,108) and antagonists which block the ACh-induced inhibition also block the stimulation-induced inhibition (106, 107). Finally, both Prosser (84) and Welsh and Slocombe (107) showed that an electrically stimulated heart released a substance which inhibited other hearts, and was thus concluded to be ACh.

It was determined very early that the pharmacology of ACh action in the Mercenaria heart differed markedly in certain respects from the classical cholinergic pharmacology of vertebrates. ACh action in vertebrates had long been noted to fall into two major categories, "muscarinic" (mimicked by muscarine and blocked by atropine) and "nicotinic" (mimicked by nicotine and blocked by d-TC; 61). Muscarinic

responses were observed in vertebrate heart and smooth muscle, and nicotinic responses were found in skeletal muscle and autonomic ganglia. Further studies showed that an additional pharmacological distinction could be made between nicotinic actions in these latter two systems. Cholinergic action in autonomic ganglia was found to be selectively mimicked by dimethylpiperazinium (DMPP) and tetramethylammonium (TMA) and blocked by hexamethonium and tetraethylammonium (TEA), while neuromuscular cholinergic activity was preferentially mimicked by phenyltrimethylammonium (PTMA) and carbamylcholine and antagonized by dihydro-β-erythroidine, benzoquinonium (see below) and, after depolarization, by decamethonium (64, 102). Additional agents which were found to specifically activate muscarinic receptors were arecoline, pilocarpine, and acetyl-β-methylcholine (methacholine; 63).

In contrast to vertebrate systems, neither dTC nor atropine were found to be very effective antagonists of the inhibitory effect of ACh on Mercenaria heart. Atropine was found to be an effective blocker only at concentrations of 10^{-3} M, with slight antagonism at 10^{-4} to 10^{-5} M, and dTC was reported to have a weak blocking action at 10^{-3} M and to potentiate slightly the effect of ACh at lower concentrations (75, 110). However, the selectivity, and for that manner, physiological meaningfulness, of antagonism is lost at these high doses (45). Among other classical cholinergic antagonists which had little or no effect on the clam heart were the neuromuscular blockers dihydro-β-erythroidine and decamethonium (75, 110). Nicotine was found to mimic the effect

of ACh at fairly low concentrations and block ACh action at high concentrations (84, 109, 110). Pilocarpine was without effect in concentrations up to 10^{-2} M (109). Carbamylcholine and methacholine both elicited ACh-like inhibition, at 80 times and 1000 times the effective concentration of ACh, respectively (110). Agents which were found to be very effective antagonists were TEA and a series of benzoquinone derivatives, in particular, the derivative named WIN 2747 or Mytolon (which is now generally referred to as benzoquinonium, BZQ) which was effective at concentrations of 5×10^{-8} M, i.e., about 1000 times as effective as TEA (75). Thus, although Mercenaria heart was affected by many of the classical cholinomimetics and cholinolytics, its reponses did not allow it to be classified into any of the three main categories of cholinergic action found in vertebrates.

Several structure-activity studies have been done (75, 108, 109, 110), in an attempt to correlate the structure of various series of ACh analogues and other compounds which act as agonists or antagonists with their activity on Mercenaria heart and from the correlations to infer what structural requirements exist for activation or blockade of the ACh receptors. These investigations have shown that in a series of alkyltrimethylammonium compounds, the amyl derivative (with the same length side chain as ACh) is by far the most effective agonist; in a series of n-ketoamyltrimethylammonium compounds, the 4-keto derivative (with the carbonyl in the same relative position as in ACh) is the most effective agonist; and in a series of tetraalkylammonium compounds, methyl groups confer cholinomimetic and ethyl groups or

longer cholinolytic activity. These results indicate that the ACh receptor of Mercenaria is indeed highly specific for a number of parameters of the ACh molecule, though they do not indicate what distinguishes this receptor structurally from the cholinergic receptors of vertebrates.

The information obtained from such structure-activity studies becomes more meaningful when the characteristics of the receptor can be compared with those of other comparable invertebrate receptors. Greenberg has investigated the activity of a variety of cholinergic-related drugs, including the same series of ACh analogues used by Welsh and Taub, on the hearts of a number of bivalves. From the results of his experiments and those of other investigators, he has attempted to determine some trends in the broad picture of ACh action in bivalve hearts (36). His conclusions include the observation that there seem to be two general types of ACh receptor in the hearts of bivalve molluscs, distinguishable by the different effects on the heart which each mediates and by differences in the pharmacology, i.e., in the relative effectiveness of various cholinergic-related drugs. In general, one receptor type mediates inhibition by ACh and is blocked by BZQ and the second mediates excitation and is blocked by dTC. Some species appear to have only one type of receptor, either inhibitory or excitatory, while other species possess both and show a biphasic response or different responses at different concentrations of ACh (35, 36, 83). This result is especially interesting in light of recent findings in gastropod molluscs of multiple cholinergic receptor types (see below).

There have as yet been no investigations into the underlying ionic mechanisms of ACh action on Mercenaria heart, although Florey and Merwin (30) have looked at the effects of K^+ ion on the hearts of two other bivalves (Protothaca staminea and Mya arenaria) which are inhibited by very low concentrations of ACh. They reported that lowering the K^+ concentration in the perfusion medium increased the sensitivity of the hearts to ACh inhibition, while raising the K^+ concentration reduced the ACh sensitivity. This is what would be expected if, as is the case in mammalian heart (39, 44, 98, 99), ACh exerted its inhibitory effect by increasing membrane permeability to K^+. The evidence presented by Florey and Merwin is not so convincing, however, since the changes in K^+ concentration by themselves had very marked effects on the spontaneous heart beat. K^+-free solution alone depressed the heart and caused eventual diastolic arrest, while the higher concentrations of K^+ which were used caused systolic arrest (30).

The intracellular studies of Irisawa and coworkers (46, 47, 48, 49, 50, 51) on the hearts of the oyster (Ostrea gigas) and mussel (Mytilus edulis) have elucidated the ionic mechanisms of ACh action in these two systems. Oyster heart is inhibited and mussel heart excited by ACh. The intracellular correlates of these two effects, as determined by Irisawa et al., are an ACh-induced hyperpolarization in the oyster heart, apparently mediated by an increase in Cl^- permeability, and an ACh-induced depolarization in the mussel heart, brought about by an increase in Na^+ permeability (51).

The final, compelling proof of a transmitter action for ACh in Mercenaria heart (and in other bivalve hearts) as well as the

determination of its exact mechanism of action await the application of the more precise techniques of intracellular recording and iontophoretic application of drugs, as were used by Irisawa et al. In the course of the use of the Mercenaria heart as a routine bioassay in the experiments in this thesis, evidence has been obtained for similarities between the mode of action of ACh on the clam heart and its action in another molluscan system, an Aplysia central ganglion, which has been well studied by intracellular techniques. By comparison with this and other similar systems, it may be possible to obtain a better understanding of cholinergic action in the bivalve heart. Out of the comparison may come, in addition, a more comprehensive picture of the action of ACh in invertebrates.

3. Cholinergic Action in Other Molluscan (Gastropod) Systems

The invertebrate systems to which the precise techniques of intracellular recording and iontophoretic drug application have been applied are the ganglia of gastropods, especially Aplysia, Helix, Cryptomphallus, Navanax, and Onchidium. The presence of very large neurons, easily penetrable and reliably identifiable, have made such ganglia an obvious choice for neurophysiological study. In these systems, ACh responses can be studied on the cellular rather than whole-organ level, and much more can be said about the ionic mechanisms underlying the responses and the selectivity of receptor activation by various cholinomimetics and blockade by cholinolytics.

Intracellular investigations on gastropod ganglia have shown that, as was true in vertebrates, there are several types of cholinergic

responses or receptors, but, as was true in bivalves, these receptors do not fit easily into the vertebrate classification. Tauc suggested a classification of the observed gastropod cholinergic responses (97), which includes "D" receptors, or those whose activation results in a depolarization of the cell and which have been found to be blocked by dTC, atropine and hexamethonium, and "H" receptors, or those which give a hyperpolarizing response to ACh and which are blocked by dTC and atropine but not hexamethonium. The D response has been shown to result from an increase in Na^+ permeability and the H response from an increase in Cl^- permeability (10, 11, 82, 90). These two types of cholinergic receptors have been reported in virtually every gastropod ganglion studied (see 33, 97 for reviews). However, several cholinergic responses have been described which do not fit the H and D classification. Levitan and Tauc (72) have reported a hyperpolarizing, Cl^--mediated inhibition in the buccal ganglion of Navanax which was not blocked by any of the cholinolytics tried, including dTC, atropine, and hexamethonium. This receptor was activated by methacholine, oxotremorine, pilocarpine and, to a lesser degree, carbamylcholine. Kehoe (57, 58) has described a type of cholinergic receptor in Aplysia pleural ganglion whose activation results in a slow hyperpolarization which is blocked selectively only by methylxylocholine (also called βTM10, hereafter abbreviated MXC), TEA and PTMA, and not by dTC, atropine, or hexamethonium.

Kehoe's unique receptor is of special interest here because of the similarities between it and the inhibitory cholinergic response

previously described in Mercenaria heart. Kehoe has demonstrated the activation of this receptor, present on the medial cells of the pleural ganglion, both by presynaptic stimulation of the "identifiable presynaptic neuron I" which innervates the medial cells and by iontophoretic injection of ACh onto the soma of the medial cells (57). In both cases, the response inverts at -80 mV, the K^+ equilibrium potential in this system, and is dependent on external K^+ concentration to the full extent predicted by the Nernst equation for a system acting as a K^+ electrode (57). Thus the response may be concluded to result from an increase in K^+ permeability.

Carbamylcholine activates this slow hyperpolarizing receptor, but not specifically, since it also activates a second, rapid hyperpolarizing cholinergic receptor (which fits into Tauc's classification as an H receptor) on the same medial cells, as well as a third type of depolarizing cholinergic receptor (a D receptor) found on anterior cells of the same pleural ganglion. Arecoline, a "classical" muscarinic agonist, is the only cholinomimetic which specifically activates only the slow hyperpolarizing receptor. Nicotine and muscarine are without effect on this receptor, while oxotremorine, methacholine and pilocarpine all have weak non-specific cholinomimetic effects on all three receptor types. BZQ antagonizes the activation of the slow receptor, but is non-specific in that it also blocks the D and H receptors (although somewhat less effectively; Kehoe, personal communication).

Kehoe has also detected the presence of the slow hyperpolarizing receptor in a number of cells in the abdominal ganglion in Aplysia

(59,60). To date, however, this receptor type has not been noted in any other preparation besides Aplysia pleural and abdominal ganglia. This may well be due to the fact that the slow hyperpolarizing response is easily obscured by the presence of the H and/or D responses on the same cell and careful investigation at a number of membrane potential levels is necessary to detect it. Moreover, in experiments in which ACh and various agonists are added to the bath, rather than applied by the well defined and highly localized method of iontophoresis, the multi-phasic nature of a cholinergic response is usually not apparent (5, 59).

It is of interest to note that, in addition to the presence of two hyperpolarizing receptors, rapid and slow, on the medial cells, Kehoe has demonstrated the presence of two and even three types of cholinergic receptors (various combinations of D, rapid H, and slow hyperpolarizing) on the same cell in the Aplysia abdominal ganglion (59). The presence on the same cell of multiple receptors to a single transmitter appears, in fact, to be a commonly observed characteristic in gastropod ganglia (5) and has been reported for 5-HT (34), dopamine (4), and glutamine (96), as well as for ACh in many other preparations (32, 72, 103, 104). In the case of multiple cholinergic receptors, it has been shown that, because of differential localization of the receptor types on the cell membrane (72), differential thresholds of activation and/or differential sensitization characteristics (104), the exact nature of the postsynaptic response can depend highly on the duration or frequency of presynaptic stimulation, or the amount and exact location of release of

iontophoretically applied ACh. As noted above, bath application of ACh in such cases can mask the presence of multiple responses. These findings regarding multiple receptors in gastropod ganglia would appear to represent the cellular correlate of the phenomenon in clam heart of dual effects of ACh on the same tissue.

Research on transmitter mechanisms, cholinergic as well as others, in the central nervous system (CNS) of certain gastropods has been very productive in elucidating the ionic and molecular mechanisms involved. It seems certain that the extension of the same type of research to other molluscan systems and to the peripheral as well as the central nervous system will be equally productive. Some of the findings of the work in this thesis point to bivalve hearts as a molluscan peripheral system which is ripe for the application of such research techniques.

MATERIALS AND METHODS

1. Chemicals, Biochemicals, Biological Specimens

Bismuth subnitrate was obtained from Allied Chemical. Chloroplatinic acid was obtained from J. T. Baker. Choline chloride, phosphomolybdic acid, acetylcholine chloride, 1-amino-2-naphthol-4-sulfonic acid (ANS), tetramethylammonium bromide, and p-dimethylaminobenzaldehyde were obtained from Matheson, Coleman and Bell. Dipicrylamine, gallamine triethiodide (flaxedil), phenyltrimethylammonium chloride and decamethonium bromide were obtained from K & K Laboratories (Division of ICN). Arecoline and methylxylocholine (βTM10) were gifts of Smith, Kline and French Laboratories. Arecoline was also obtained from Sigma. Serotonin creatinine sulfate, d-tubocurarine chloride, atropine, pilocarpine, neostigmine (prostigmine) bromide, physostigmine bromide (eserine), ninhydrin, acetylcholine chloride, butyrylcholine chloride, carbamylcholine chloride, laurylcholine chloride, propionylcholine chloride, succinylcholine chloride and nicotine were obtained from Sigma. Caffeine, homarine hydrochloride (N-methylpicolinic acid), acetylthiocholine iodide, and 5, 5'-dithiobis-(2-nitrobenzoic acid) were from Aldrich Chemical Company. Methysergide (UML-491) was a gift of Sandoz. Tetraethylammonium bromide and N-methylpyridinium were from Eastman. Benzoquinonium (WIN 2747, Mytolon) was a gift of Sterling-Winthrop Research Institute, Rensselaer, N. Y. Dithiothreitol (Cleland's Reagent) and muscarine hydrochloride were obtained from

Calbiochem. Hexamethonium chloride dihydrate and neurine bromide were from Mann Research Laboratories. Folin & Ciocalteau Reagent was from Steri-Kem Incorporation. Ammonium molybdate was obtained from Mallinckrodt Chemical Works. Bungarus multicinctus venom was from Miami Serpentarium. Tetrodotoxin was obtained from Sankyo, Tokyo. Instant Ocean synthetic sea water salts were from Aquarium Systems, Incorporated, Eastlake, Ohio.

Trypsin (bovine pancreas), DNAse I (bovine pancreas), and RNAse A (bovine pancreas) were obtained from Sigma. Butyryl cholinesterase (horse serum) was from Worthington Enzymes. Pronase-P (B grade, Streptomyces griseus) was obtained from California Corporation for Biochemical Research. Chymotrypsinogen was from Armour Research Division.

Sephadex G-50, DEAE-Sephadex (A-25), CM-Sephadex (C-25) and SP-Sephadex (C-25) were obtained from Pharmacia Fine Chemicals. The following precoated 20 × 20 cm glass TLC plates were obtained through Brinkmann Instruments, Incorporated: Cellulose MN300, 0.5 mm thick; Alox-100 UV_{254}, 1.0 mm aluminum oxide; EM silica gel F-254, 0.5 mm thick. 5 × 10 cm glass plates, precoated with 0.25 mm silica gel F-254 (Silplate-P5F-22) and Polygram, Alox N/UV_{254}, alumina-coated plastic strips, 0.2 mm thick, were also from Brinkmann Instruments. Eastman Chromagram sheets, 13255 cellulose with fluorescent indicator, 20 × 20 cm, 0.16 mm thick, were obtained from Eastman Organic Chemicals. Desaga cellulose acetate connecting strips for TLE were obtained through Brinkmann Instruments.

Mercenaria mercenaria were purchased from New England Live Lobster, Sunset Beach, California. Conus californicus and Aplysia californica were supplied by Pacific Biomarine Supply Company, Venice, California. Rana pipiens were obtained from Schettle Biologicals, Stillwater, Minnesota, from Los Angeles Biologicals, Gardena, California (now Circle Scientific, Long Beach, California), and from G. W. Fish and Poultry Market, Los Angeles, California.

2. Extraction and Purification of Conusine

Conus californicus of 20-32 mm in length were kept in aerated, filtered, 14°C sea water without feeding (from 3 days to several months) until use. The shell of the snail was cracked in a vice clamp and the foot and viscera cut away. The remainder of the organism was pinned to a small dissecting dish and the venom duct dissected out under a dissecting microscope, using the dissection procedure outlined by Hermitte (40) as a guide. The venom ducts of a number of Conus were homogenized in methanol in a ground glass homogenizer and centrifuged at 12, 000 × g for 10 min to pellet insoluble material. The pellet was washed twice with methanol and centrifuged, and the combined methanol supernatants were evaporated down at 40°C under a stream of filtered air. The residue was weighed, dissolved in 20 mM ammonium acetate buffer, pH 8.3, and loaded on a Sephadex G-50 column, 1.5 cm × 100 cm, equilibrated in the same buffer. Fractions were monitored both for bioactivity on the excised clam heart and for absorption at 280 nm. Since the activity eluted reliably within a large

peak in A_{280}, the A_{280} profile could be used for locating the conusine without bioassay. The active fractions were pooled and lyophilized and the residue dissolved in a small amount of methanol. This sample solution was loaded, by repeated spotting, on a 0.5 mm silica gel thin layer chromatography (TLC) plate, 20 cm × 20 cm, and developed for $3\frac{1}{2}$-4 hrs in either ethanol-water, 7:3, or ethanol-ammonium hydroxide-water, 7:2:1. The developed chromatogram was dried in a hood, stained in an iodine vapor-saturated tank to visualize the bands, which were marked in pencil, and the iodine then allowed to sublime away. The band at the origin contained the conusine, as determined by bioassay of extracts of the TLC plate. This band was extracted by scraping the silica gel off the TLC plate into a glass centrifuge tube, mixing well with 7.0 ml distilled water or 1.0 M ammonium acetate, and centrifuging at 27,000 × g for 15 min. The silica gel pellet was washed twice with 7.0 ml aliquots of the extracting solvent and centrifuged, and the combined supernatants were lyophilized. The residue was dissolved in a small amount of methanol and loaded by repeated spotting onto a 0.5 mm cellulose TLC plate, 20 cm x 20 cm. This second semi-preparative TLC was developed for 3-$3\frac{1}{2}$ hrs in ethanol-water, 7:3, dried in a hood, and stained with iodine vapor. Of the two detectable bands, the one corresponding to an R_f of 0.72 was conusine, as determined by bioassay on clam heart. This band was marked in pencil, and the iodine allowed to sublime away. The marked band of cellulose was scraped from the TLC plate, extracted three times with water and the supernatants lyophilized.

3. Analytical Thin Layer and Paper Chromatography, Spray Reagents

At various stages of the purification, the homogeneity of the conusine sample was checked by paper chromatography and analytical TLC with several adsorbents. Whatman No. 1 paper was used for paper chromatograms. Three different substrates were used for analytical TLC: 0.16 mm cellulose, 0.2 mm alumina, and 0.25 mm silica gel. Chromatograms were developed in three different solvent systems:

Solvent system A ethanol-ammonium hydroxide-water, 7:2:1

Solvent system B butanol-glacial acetic acid-water, 4:1:1

Solvent system C ethanol-water, 7:3.

The chromatograms were visualized by iodine vapor staining, which proved to be the best general indicator (sensitivity 1-10 μg), detecting spots which were not detected by UV fluorescence quenching or H_2SO_4 charring. After sublimation of the iodine, chromatograms were then sprayed with one of a variety of more specific indicator reagents, prepared as described below.

Ninhydrin (detects aliphatic or alicyclic primary amino groups; sensitivity--1-2 μg). 0.5 g ninhydrin was dissolved in 100 ml acetone. The chromatogram was sprayed with this solution and heated on a hot plate, or in a 105°C oven, for 5 min (19).

Ehrlich's Reagent (detects indolealkylamines; sensitivity--1-3 μg). A 2% solution of p-dimethylaminobenzaldehyde was prepared in 6 N HCl (19).

Dragendorf's reagent (detects alkaloids, tertiary amines and quaternary ammonium compounds; sensitivity--0.2-5 μg). There are a number of modifications of this widely used spray reagent, each modification imparting slightly different specificity to the reagent. The following modifications were used in this thesis.

Munier Modification. Solution A was prepared by dissolving 17 g bismuth subnitrate plus 200 g tartaric acid in 800 ml water. Solution B was prepared by dissolving 160 g potassium iodide in 400 ml water. The color reagent was prepared by mixing 25 ml solution A with 25 ml solution B, adding 500 ml water and dissolving in this solution 100 g tartaric acid (19, 118).

Munier and Macheboeuf Modification. Solution A was prepared by dissolving 0.85 g bismuth subnitrate in a mixture of 10 ml glacial acetic acid and 40 ml water. Solution B was prepared by dissolving 8 g potassium iodide in 20 ml water. The color reagent was prepared by mixing 5 ml solution A with 5 ml solution B, adding 20 ml glacial acetic acid, and diluting to 100 ml with water (118).

Theis and Reuther Modification, further modified by Vágùjfalvi. Solution A was prepared by dissolving 2.6 g basic bismuth carbonate and 7.0 g dry sodium iodide in 25 ml boiling glacial acetic acid. The solution was allowed to stand 12 hours and filtered. Solution B was prepared by adding 8 ml ethyl acetate to 20 ml of the clear filtrate of solution A. The color reagent was prepared by mixing 10 ml solution B, 25 ml glacial acetic acid, and 60 ml ethyl acetate (19, 118).

Iodoplatinate (detects tertiary amines, quaternary ammonium compounds, and alkaloids; sensitivity--10-50 μg). 5 ml of 5% aqueous platinic chloride was mixed with 45 ml of 10% aqueous potassium iodide and added to 100 ml water (19, 118).

Dipicrylamine (detects quaternary ammonium bases; sensitivity--~5 μg). A 0.4% solution of dipicrylamine (DPA) was prepared in 50% aqueous ethanolic solution (7, 118).

Phosphomolybdic acid (detects lipids, choline and choline esters; sensitivity--5-10 μg). A 10% solution of phosphomolybdic acid was prepared in absolute ethanol. The chromatogram was heated (to ~160°-180°C) on a hot plate and sprayed with this reagent immediately (19).

Hydroxamate-Ferric Chloride (detects carboxylic esters; sensitivity 0.5-2 μg). Solution A was prepared by dissolving 20 g hydroxylamine hydrochloride in 50 ml water and diluting to 200 ml with 95% ethanol. This solution was then mixed 2:1 with solution B, an ethanolic solution of potassium hydroxide, and the potassium chloride precipitate was filtered off. Solution C was prepared by dissolving 10 g ferric chloride ($FeCl_3 \cdot 6H_2O$) in 20 ml of 10 N HCl

and shaking this solution with 200 ml ether until a homogeneous suspension was obtained. The chromatogram was sprayed with solutions A + B, allowed to dry, and then sprayed with solution C (7).

4. Thin Layer Electrophoresis

Both analytical and preparative thin layer electrophoreses (TLE's) were run in the Desaga/Brinkmann Thin Layer Electrophoresis Double Chamber, using a Heathkit Regulated High Voltage Power Supply, Model IP-17. Analytical electrophoreses were run on 0.16 mm thick, 20 cm long, Eastman cellulose chromagram sheets, using Desaga cellulose acetate connecting strips to make contact between the cellulose sheets and the buffer reservoirs. The following buffer systems were used: 350 ml pyridine + 3.5 ml glacial acetic acid to 1.0 liter with water, pH 6.5; a series of 50 mM ammonium acetate buffers, with pH's of 8.5, 9.0, 9.5, and 10.0, and a series of 50 mM triethylamine-acetic acid buffers with pH's of 10.5, 11.0, and 11.5. Preliminary analytical electrophoreses were run in each buffer by loading the sample spots in the middle of two pre-electrophoresed cellulose sheets and electrophoresing at 400 volts for 15 min and 30 min, respectively. The TLE sheet was air-dried in a hood and stained in an iodine vapor tank. From this preliminary run it was determined

whether, at the pH of the given buffer, the sample should be loaded at the anode end or cathode end for a longer electrophoresis. Longer analytical electrophoreses were run at 400 volts for 45 and 60 min, respectively. These TLEs were similarly air-dried and iodine-stained.

A preparative electrophoresis of conusine purified on silica gel TLC was performed on a 0.5 mm cellulose-coated glass TLC plate (Brinkmann cellulose MN 300, 20 cm x 20 cm). The TLE was electrophoresed at 400 volts in 50 mM ammonium acetate, pH 8.3, for two successive periods of 45 min and 30 min, with air-drying and iodine-staining of the TLE after each run. There was only one detectable band in the TLE, other than impurities at the anode and cathode ends which were detectable on an unloaded control plate electrophoresed under identical conditions. This band, along with the bands of impurities, was extracted by scraping the cellulose into a centrifuge tube, mixing with 7.0 ml distilled water, and centrifuging at 27,000 x g for 15 min. The cellulose pellet was washed twice with 7.0 ml aliquots of water and recentrifuged, and the combined supernatants were lyophilized and bioassayed on clam heart.

5. Spectra

Ultra-violet spectra of silica gel TLC-purified conusine in methanol solution were run in a Cary 14 UV-visible spectrophotometer.

Infrared spectra of KBr pellets of cellulose TLC-purified conusine were obtained on a Perkin-Elmer 257 infrared grating spectrophotometer.

^{1}H spectra of cellulose TLC-purified conusine were obtained (in collaboration with Dr. Ken Reed) using a Varian XL-100-15 NMR spectrometer operating at 100 MHz in the Fourier transform mode and locked on the ^{2}H resonance of the solvent (d_6-DMSO or D_2O). The ambient temperature in the probe was 29°C. To eliminate the dynamic range problems resulting from the residual protons of the solvent, the ^{1}H solvent peak was saturated using the Varian Gyrocode decoupler. In spectra obtained in D_2O, ethanol was added to the sample for purposes of calibration.. The chemical shift values of conusine were then calculated relative to the methyl proton peaks of the ethanol.

A volatile derivative of silica gel TLC-purified conusine was prepared for gas chromatography and mass spectral analysis (in collaboration with Dr. D. J. Jenden) by N-demethylation with benzenethiolate (53). The resulting derivative was chromatographed on a gas chromatograph column coupled to a mass spectrometer, and the mass fragmentogram of the effluent peak was determined.

6. Denaturation Experiments

The following experiments were performed on conusine to determine its stability to heat, extreme pH conditions, sulfhydryl reduction, and enzymatic degradation. It was difficult to quantitate the amounts of conusine used in all the following experiments, since the samples used had been purified only as far as the G-50 fractionation step. The dry weight of the sample reflected, therefore, the presence of other venom components as well as residual ammonium acetate. For the purposes of these experiments, it was sufficient to compare the bioactivity of a treated aliquot of conusine with that of an untreated aliquot from the same solution, without knowing the concentration of conusine present.

Heat--100 μl aliquots of a solution of conusine in artificial sea water (ASW) were treated with the following temperature regimens: 23°C for 24 hrs; 37°C for 10 min, 20 min, 30 min and one hr; and 100°C (in a boiling water bath) for 30 min and one hr. These heat-treated aliquots were then bioassayed on clam heart and compared to the untreated conusine solution.

pH--Two 10 μl aliquots of a solution of conusine in ASW were added to 90 μl ASW brought to pH 2.0 with concentrated HCl and to 90 μl ASW brought to pH 10.0 with 1 M NaOH, respectively. The mixtures were allowed to sit overnight at 10°C and then bioassayed on clam heart. The activity of the pH-treated conusine was compared to that of a 1:10 dilution of untreated conusine. The effects of pH 2 ASW and pH 10 ASW were also tested on clam heart by adding these

pH-altered solutions in amounts equal to those used in the bioassay of the pH-treated conusine.

In addition to these pH studies, conusine was heated in the presence of strong acid and strong base. Two 10 μl aliquots of conusine in ASW were added to 90 μl of 1.0 M HCl and 90 μl of 1.0 M NaOH, respectively. These solutions were covered with Parafilm and placed in a boiling water bath for one hour. The acidic conusine solution was neutralized with NaOH and the basic conusine solution was neutralized with HCl before bioassay on clam heart. The activity of acid- and alkali-treated conusine was compared to that of the appropriate dilution of untreated conusine.

Dithiothreitol (Cleland's Reagent)--1.54 mg of dithiothreitol (DTT) was dissolved in 100 μl of ASW, to give a stock solution of 100 mM. 10 μl of this solution was added to 80 μl ASW and 30 μl conusine in ASW solution, to give a final DTT concentration of 10 mM and a conusine dilution of 1:10. The mixture was incubated for 2 hrs at 37°C. The bioactivity on clam heart of DTT-treated conusine was compared with that of untreated conusine. The effect of 10 mM DTT on clam heart was also checked by adding DTT-ASW in amounts equal to those used in the bioassay of DTT-treated conusine.

7. Enzymatic Incubations of Conusine

The basic format of all the following enzymatic treatments of conusine is the same. An aliquot of a conusine solution was incubated with a given enzyme in its required buffer system, a second aliquot of

the conusine solution was incubated for the same length of time and under the same conditions with the buffer system minus the enzyme, and a third aliquot of the conusine was untreated and only appropriately diluted. Incubations were ended by placing the mixture on ice and then at -20°C until bioassay, which was performed from immediately after incubation up to a week later. The bioactivities of the treated, control, and untreated conusine solutions were tested on clam heart, along with the effect of the buffer solutions plus enzyme but minus conusine. The concentration of the original conusine solution was chosen to be such that it could be diluted as necessary in the enzyme incubation mixture, and then further diluted for bioassay to an extent sufficient to minimize direct effects of the enzyme on the clam heart.

Trypsin--1.0 mg trypsin (12 BAEE units/mg) was dissolved in 100 μl of 0.001 M HCl to give a 10 mg/ml stock solution. 30 μl of this stock enzyme solution was added to 240 μl of 0.046 M Tris buffer, pH 8.1, containing 0.0115 M $CaCl_2$ (116) and 30 μl of an ASW solution of conusine, to give a final enzyme concentration of 1 mg/ml and a conusine dilution of 1:10. This mixture was incubated for 2 hrs at 37°C.

Pronase--1.0 mg pronase-P (45 units/mg) was dissolved in 100 μl of the following buffer: 0.05 M sodium phosphate, pH 5.5, containing 10 mM $CaCl_2$ and 1 mM $CoCl_2 \cdot 6H_2$ (81). 30 μl of this stock enzyme solution was added to 240 μl of the same buffer and 30 μl of an ASW solution of conusine to give a final enzyme concentration of 1 mg/ml and a conusine dilution of 1:10. This mixture was incubated for 2 hrs at 40°C.

Chymotrypsin--1.0 mg chymotrypsin was dissolved in 100 μl of 0.001 M HCl to give a stock solution of 10 mg/ml. 30 μl of this stock solution was added to 240 μl of 0.08 M Tris buffer, pH 7.8, containing 0.1 M $CaCl_2$ (116), and 30 μl aqueous conusine solution to give a final enzyme concentration of 1 mg/ml and a conusine dilution of 1:10. The mixture was incubated for 2 hrs at 37°C.

Pepsin--1.0 mg pepsinogen was dissolved in 100 μl of 0.001 M HCl. 30 μl of this stock solution was added to 240 μl of the incubation medium, ASW brought to pH 2.0 with 0.01 M HCl (116), plus 30 μl aqueous conusine solution, to give a final enzyme concentration of 1.0 mg/ml and a conusine dilution of 1:10. This mixture was incubated for 2 hrs at 37°C.

DNAse--1.0 mg DNAse I (820 units/mg) was dissolved in 1.0 ml water. 30 μl of this stock solution was added to 240 μl of a 0.05 M sodium acetate buffer, pH 5.0, containing 0.05 M $MgSO_4$ (116) and 30 μl aqueous conusine solution, to give a final enzyme concentration of 100 μg/ml and a conusine dilution of 1:10. This mixture was incubated for 2 hrs at 37°C.

RNAse--1.0 mg RNAse-A (75 units/mg) was dissolved in 100 μl water. 30 μl of this stock solution was added to 240 μl of 0.05 M sodium acetate buffer, pH 5.0 (116) and 30 μl of an ASW solution of conusine, to give a final enzyme concentration of 1.0 mg/ml and a conusine dilution of 1:10. This mixture was incubated for 2 hrs at 37°C.

Acetylcholinesterase--AChE (present in membrane fragments isolated from Torpedo electroplax in 10 mM sodium phosphate-0.4

M NaCl, pH 7.4 and purified by several differential centrifugations and a zonal centrifugation on a 25-55% sucrose gradient, 86) was assayed for cholinesterase activity, using the spectrophotometric assay for cholinesterase (see below). 50 μl of a 1:100 dilution of the stock AChE solution, an amount of enzyme sufficient to hydrolyze 100 μmoles of acetylthiocholine in one minute, was added to 1.0 ml of 0.1 M sodium phosphate buffer, pH 7. This enzyme solution was added to a vial containing the residue from a 525 μl aliquot of a methanolic conusine solution and the mixture was allowed to incubate for one hour at 23°C. Incubation was terminated in this case by heating the mixture for 5 min at 100°C in a boiling water bath.

Butyrylcholinesterase (BuChE)--2.0 mg BuChE (4-6 units/mg) was dissolved in 100 μl 0.1 M sodium phosphate buffer, pH 7, to give a stock solution of 20 mg/ml. This solution was assayed for esterase activity using the spectrophotometric assay for AChE (see below). 25 μl of this stock solution was added to 1.0 ml of 0.1 M sodium phosphate buffer, pH 7.0, to give an enzyme solution capable of hydrolyzing 93 μmoles of acetylthiocholine per min. This enzyme solution was added to a vial containing the residue from a 525 μl aliquot of a methanolic conusine solution, and the mixture was allowed to incubate 1 hr at 23°C. Again, incubation was terminated by heating the mixture for 5 min at 100°C in a boiling water bath.

8. Ion Exchanger Studies

The pooled active fractions from G-50 fractionated Conus venom were divided into four equal aliquots, two of which were lyophilized.

To one of the unlyophilized aliquots, in 20 mM ammonium acetate, pH 8.3, was added a slurry of 5 ml (0.63 g dry weight = 2.2 milliequivalents) of DEAE-Sephadex, equilibrated in the same buffer. To the residues of the two lyophilized fractions were added, respectively, a slurry of 5.0 ml (0.5 g dry weight = 2.7 milliequivalents) of swollen CM-Sephadex and a slurry of 5.0 ml (0.7 g dry weight = 1.6 milliequivalents) of SP-Sephadex, both equilibrated in 20 mM ammonium acetate, pH 5.0. The final aliquot in 20 mM ammonium acetate, pH 8.3, was reserved untreated for bioassay. The three ion exchanger-toxin mixtures were diluted with the appropriate buffer to 7.5 ml and then shaken on a Buchler test tube shaker for 12 hrs. The mixtures were then centrifuged at 12,000 x g for 10 min and the supernatants pipetted off and lyophilized for bioassay. The pellets of ion exchanger were then resuspended in 1.0 M ammonium acetate buffer, pH 8.3 in the case of the DEAE-Sephadex and pH 5.0 in the cases of CM- and SP-Sephadex. These mixtures were again shaken 12 hrs, centrifuged and supernatants pipetted off and lyophilized for bioassay. The activity on clam heart of the low salt and high salt supernatants, redissolved in ASW, was compared with that of the untreated aliquot, also lyophilized and redissolved in ASW.

9. Enzyme Assays

Acetylcholinesterase--The spectrophotometric assay of Ellmann et al. (22) for cholinesterase activity was employed to determine the activity of the AChE and BuChE used in the denaturation experiments, as well as to determine the effect of conusine on the activity of AChE.

To 150 ml of 0.1 M sodium phosphate buffer, pH 8, was added 5.0 ml of a 4.0 mg/ml solution of DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid)) in 0.1 M sodium phosphate buffer, pH 8, and 1.0 ml of an aqueous acetylthiocholine solution, 65 mg/3 ml. 3.0 ml of this mixture was pipetted into the cuvette of a Gilford Model 240 UV-visible spectrophotometer and the machine nulled to this solution. Aliquots of AChE were added at T_0 and the absorbance at 412 nm was read on a Gilford digital read-out every 10 sec for 5 to 6 min. From the rate of increase in absorbance, due to reduction of DTNB by thiocholine, the rate of hydrolysis of acetylthiocholine, and thus the activity of the enzyme aliquot, was determined.

The effect of conusine on the activity of both AChE from Torpedo electroplax and AChE from Mercenaria heart was investigated. AChE was isolated from Torpedo electroplax as described above. The clam heart AChE used in this assay was extracted from 18 clam hearts homogenized in 1.0 M NaCl- 1.0 M sodium phosphate buffer, pH 8. The homogenate was centrifuged for 15 min at 18, 800 $\times$ g, the pellet washed once in buffer, and the supernatants combined to give a total volume of 0.7 ml.

20 μl or 30 μl of a conusine solution was added to the 3.0 ml substrate mixture described above, to give a final concentration of conusine which was very active on clam heart. To this substrate mixture plus conusine were added 5, 10, 15 and 20 μl aliquots of AChE from Torpedo electroplax and 50 μl aliquots of clam heart AChE.

The effect of eserine and neostigmine on cholinesterase activity as monitored by this spectrophotometric assay was demonstrated by adding 30 μl of 10^{-2} M eserine or neostigmine to the 3.0 ml substrate mixture, to give a final esterase inhibitor concentration of 10^{-4} M, and then adding either Torpedo AChE or clam heart AChE. The activities of the two esterases in substrate mixture, substrate mixture + conusine, and substrate mixture + eserine or neostigmine were all compared.

ATPase--The following assay, using membrane bound ATPase from eel electroplax, was performed to determine the effect of conusine on ATPase activity. 10 μl aliquots of the ATPase solution were added to vials containing the following substrate incubation mixture: 50 μl of 50 mM ATP, 25 μl of 200 mM $MgCl_2$, 2.5 μl of 200 mM EDTA, 250 μl of 200 mM imidazole, pH 7.4, 100 μl 0f 1.0 M NaCl, 100 μl of 0.2 M KCl, and 472.5 μl water (sufficient to give 1.0 ml total volume). The cocktail plus enzyme was mixed well and incubated for 10 min at 37°C. The reaction was stopped by adding 1.0 ml 10% ice-cold trichloroacetic acid to each vial and mixing well. Precipitated protein in the mixture was pelleted by centrifuging at 100 × g for 10 min and the released phosphate was determined as phosphorous by the method of Fiske and SubbaRow (28). To 1.0 ml of each supernatant were added 1.0 ml of 5.0 N H_2SO_4, 1.0 ml of 2.5% ammonium molybdate, 0.1 ml of ANS reagent (200 mg ANS + 1.2 g sodium bisulfite + 1.2 g sodium sulfite ground together and dissolved in water at a concentration of 25 mg/ml), and 7.9 ml water. After 20 min the absorbance at 660 nm was read. A calibration curve was constructed

by adding known aliquots of an inorganic phosphorous standard to the substrate incubation cocktail. Using this calibration curve, the activity of the ATPase aliquot was determined from the A_{660}. This assay was performed, adding 30 μl and 75 μl aliquots of conusine (1 mg/250 μl of G-50 fractionated sample) to the substrate cocktail and incubating for periods of 10.5 and 20 min. The activity of the ATPase in the presence of conusine was compared to that in the absence of conusine.

10. Clam Heart Bioassay

The spontaneously beating excised ventricle of Mercenaria mercenaria comprised the bioassay system used to monitor conusine activity throughout the purification and biochemical investigations. The bioassay set-up and procedure were essentially those described by Welsh (108) and Florey (29). Conusine at very low concentrations depressed and at higher concentrations abolished the spontaneous beating of the heart.

Clams were stored for up to 8 months in a large tank of aerated, filtered, 14°C sea water. The dorsal shell of the clam was cracked near the umbo with a hammer, allowing the adductor muscles to be cut with a scalpel. The entire dorsal shell was then removed. The pericardium was opened and the ventricle removed by cutting the intestine, which runs through the ventricle, on either side of its exit from the heart and by cutting through the left and right auricles, leaving portions of auricular tissue attached to the ventricle. The

ventricle was hooked with small wire hooks through either side near the auricular junction, and attached on one side to a stationary $4\frac{1}{2}$ inch 18 gage or $3\frac{1}{2}$ inch 22 gage syringe needle and on the other side to a wire leading to a Grass FT03 force transducer. The syringe needle with heart attached was lowered into a 2.5 ml plastic syringe, cut off at the 2.0 ml mark, which served as a calibrated bath, and the force transducer was positioned above the heart so as to exert a slight amount of tension (approximately .1-.2 g) on the ventricle. The bath was filled to the 1.0 ml mark with ASW from a 50 ml plastic syringe which served as a calibrated reservoir and which was fitted with Tygon tubing and a Teflon stopcock to allow controlled addition of ASW to the bath. The bath syringe itself was also fitted with Tygon tubing and a Teflon stopcock to allow controlled emptying of the bath (see Figure 3).

Heart contractions were monitored on either a Grass Model 79C polygraph, equipped with a Grass Model 7 DAE polygraph D.C. driver amplifier and a Grass Model 7 P1B low level D.C. pre-amplifier and powered by a Grass RPS 111 regulated power supply, or on a Gilson Model ICT-5H polygraph equipped with an IC-MP module.

The bath volume was maintained at 1.5 ml and drugs were diluted into the bath by means of Corning glass micropipettes. Changes in bath solution were accomplished by simultaneously opening both the reservoir stopcock and the bath stopcock, thus maintaining continuous immersion of the heart during bath solution changes. As has been noted by other investigators (41), the O_2 requirements of the clam heart appeared to be virtually negligible. The heart did not require

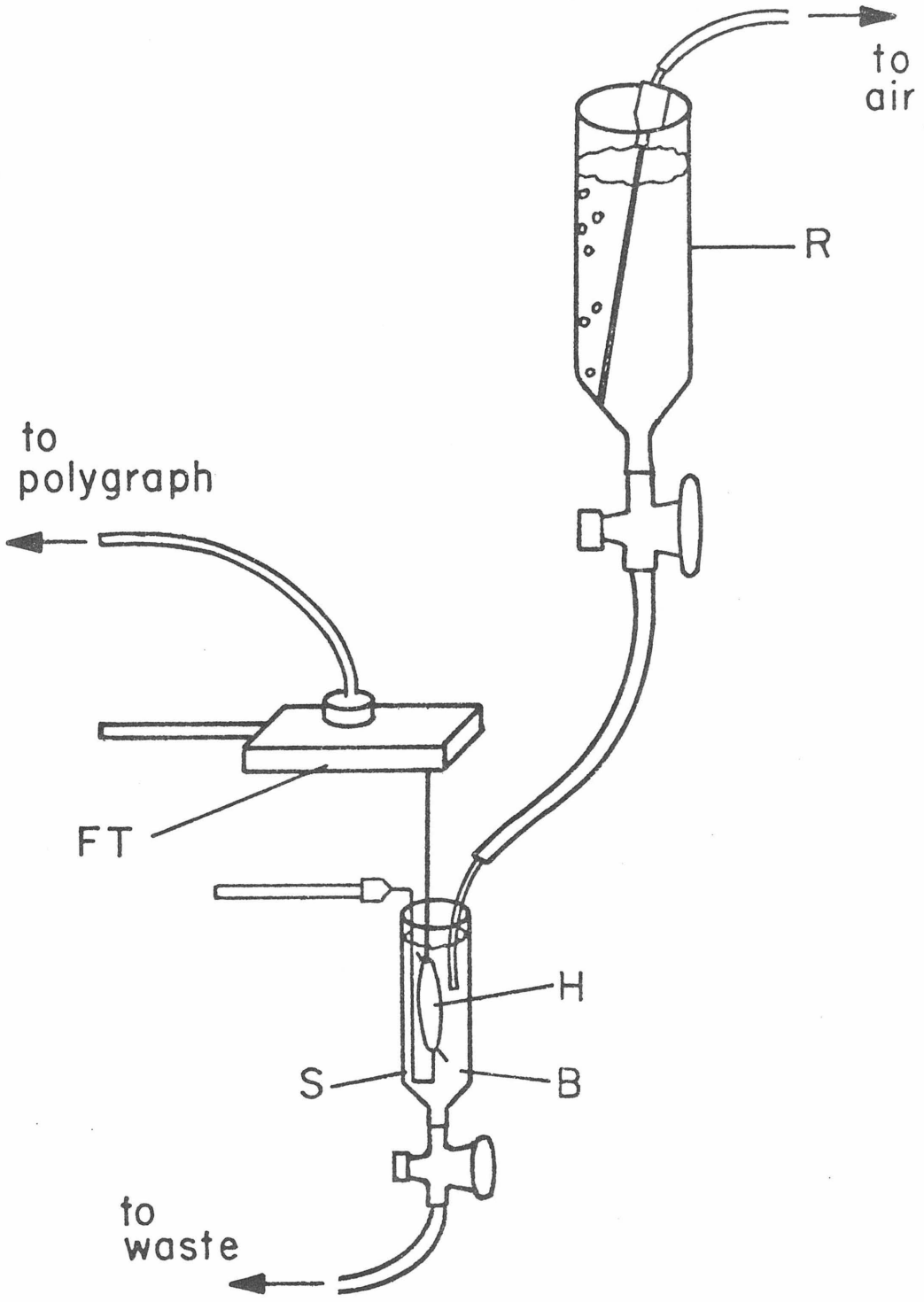


Figure 3. Set-up used in clam heart bioassay. B, bath; FT, force transducer; H, heart; R, reservoir; S, syringe needle.

direct aeration of the bath for regular, long-term spontaneous beating, and since such aeration, via the syringe needle, tended to mechanically disturb the heart and give a high background noise on the polygraph trace, aeration of the bath was not performed. ASW in the reservoir was vigorously aerated with filtered air via a 14 gage laboratory cannula.

The effect of temperature on the spontaneous beat and on the ACh response of the clam heart has been reported by Wait (105). At temperatures higher than the sea water temperatures (~ 14°-15°C) at which the clams live, the heart continues to beat regularly, though at a slightly faster rate than normal. Sensitivity to ACh, however, decreases as the temperature increases. Wait has reported that the amount of ACh required at 30°C to effect 50% inhibition is 100 times greater than that required at 10°C. Routine bioassays and other preliminary experiments were performed at ambient temperatures, from 20°C to 23°C, or sometimes at slightly lower temperatures, 16°-20°C, due to refrigeration of the ASW. Pharmacological experiments performed to determine the effect of various cholinergic agonists on the heart and of antagonists on the ACh and conusine responses of the heart were performed with both the reservoir and bath syringes cooled to 15°C with water jackets fashioned from Tygon tubing coiled about the syringes and connected to a Lauda refrigerator water pump.

The heart was bathed in Instant Ocean, in sea water (SW) or in artificial sea water (ASW) of the following composition: Per liter of solution, 27.26 g NaCl (470 mM), 0.745 g KCl (10 mM), 1.109 g $CaCl_2$

(10 mM), 10.88 g $MgCl_2 \cdot 6H_2O$ (53.5 mM), 10 mM Tris to pH 8.0 (85). In experiments in which the ionic composition of the ASW was altered, the following formulas were used: K^+-free ASW--same as for ASW, except no KCl added; $2 \times K^+$ ASW--same as for ASW, except with 1.49 g KCl (20 mM) per liter; Cl^--free ASW--per liter of solution, 33.38 g Na_2SO_4 (235 mM), 0.87 g K_2SO_4 (5 mM), 1.36 g $CaSO_4$ (10 mM), 6.44 g $MgSO_4$ (53.5 mM), and 103.88 g sucrose (303.5 mM) to maintain proper osmolarity.

11. Aplysia Pleural Ganglion Cells

In experiments performed with Dr. JacSue Kehoe at Cold Spring Harbor Laboratories, the effects of both iontophoretically-applied and bath-applied conusine were observed on two types of cells in the pleural ganglion of Aplysia. The effect of bath-applied conusine alone was observed on a third type of cell. The procedures and techniques used were the same as those reported previously by Kehoe (57, 58, 59).

The left pleural and pedal ganglia were isolated from Aplysia californica, along with the associated nerves and connectives, and were pinned to a glass chamber lined with a silicone resin (Elastomere, RTV 141, Rhône Poulenc) in which a two ml depression had been molded. The connective tissue was removed from the dorsal surface of the pleural ganglion, allowing impalement of the cells.

Double barrelled microelectrodes were pulled on a de Fonebrune microforge and filled with 0.6 M K_2SO_4. One barrel was used for recording in conjunction with a unity gain WPI M-4A electrometer and

a Brush Mark 280 rectilinear pen recorder. The second barrel was used for controlling the level of the membrane potential and for determining changes in membrane resistance. Single barrelled microelectrodes pulled with the de Fonebrune microforge were used for iontophoretic application of either ACh or conusine.

Bath application of conusine was accomplished by adding conusine dissolved in 1.0-1.5 ml sea water to 0.5 ml sea water in the bath, to give a final concentration equal to that effective on the clam heart. Experiments were conducted at ambient temperature in artificial sea water of standard composition (57).

Both the medial cells of the pleural ganglion (which show a two component hyperpolarization in response to presynaptic cholinergic stimulation as well as to iontophoretic ACh application) and the anterior cells (which are depolarized by ionophoretically applied ACh and by presynaptic cholinergic stimulation) were impaled with double-barrelled microelectrodes and their responses to iontophoretically applied conusine were recorded at a series of preset membrane potentials. The effect of bath applied conusine was recorded in these same two cell types, as well as in a third, presumably cholinergic, cell--the identifiable presynaptic neurone I (IPN I). IPN I, which synapses on the medial and anterior cells, responds to ACh application with a single component, rapid hyperpolarization.

The I-V curve of a medial cell before and after bath application of conusine was determined by injecting current pulses (at different preset membrane potentials) through one barrel of the double barrelled

microelectrode and measuring the resulting potential change with the second barrel.

Finally, the effects of bath-applied 10^{-3} g/ml methylxylocholine, 10^{-3} g/ml TEA, and 10^{-4} g/ml neostigmine (prostigmine) were observed on the medial cell response to iontophoretic application of conusine.

12. Frog Heart and Rectus Abdominis

The effect of conusine was investigated on two vertebrate preparations--the frog heart, a muscarinic muscle, and the frog rectus abdominis, a nicotinic muscle. The set-ups used in both of these assays were essentially the same as that used in the clam heart bioassay.

In the case of the frog heart, the heart was cannulated using a 2 mm, U-shaped glass cannula and was continuously perfused with aerated frog Ringer of the following composition: per liter of distilled water, 6.5 g NaCl (0.11 M), 140 mg KCl (1.9 mM), 120 mg $CaCl_2$ (1.2 mM), 200 mg $NaHCO_3$ (2.4 mM). The cannula served also as the lower, stationary support for the heart which was immersed in a 5.0 ml bath containing frog Ringer. The tip of the ventricle was hooked with a small wire hook and attached to a Grass FT03 force transducer. Drugs were added to the bath and the bath solution was changed by rinsing with Ringer from a reservoir, in the same manner as in the clam heart bioassay.

In the case of the rectus abdominis, a strip of the muscle was hooked on either end, in the same fashion as the clam heart, attached to a force transducer and suspended in a 2.5 ml bath.

All experiments were performed at ambient temperature (20-23°C). Contractions of the heart were recorded with a Gilson Model ICT-5H polygraph and contractions of the rectus abdominis were recorded with a Grass Model 79C polygraph.

RESULTS

The name conusine has been given to the neuroactive component from Conus venom which was isolated and investigated in these studies. The purification and chemical/biochemical characterization of conusine proceeded simultaneously with a characterization of its physiological activity, and these two aspects of the investigation are interrelated to a certain degree. For the sake of clarity, however, these two aspects are discussed separately in two sections below. A set of experiments which deal with new findings on the pharmacology of the clam heart are reported in a third section.

1. Chemical and Biochemical Properties of Conusine

The initial step in the purification of conusine from Conus venom was fractionation on a Sephadex G-50 column. The resulting elution profile, as monitored by absorption at 280 nm and by protein determination is shown in Figure 4. The conusine-containing fractions were located by clam heart assay (see Methods and part 2 of Results, below) of aliquots of fractions throughout the elution profile. "Activity profiles", as shown in Figure 4, were constructed from the results of such bioassays. Each fraction was assigned a value of bioactivity from - to ≡, which was determined by a number of parameters (as described in the legend to Figure 4), including the degree to which the amplitude and frequency of the clam heart beat was reduced by that fraction, the rate at which inhibition progressed, and the rate of recovery of the heart from inhibition. Figure 5 shows some sample traces from the clam heart bioassay of G-50 fractions, to illustrate the basis for the construction of the activity profile.

As can be seen from Figure 4, the peak in bioactivity does not correspond to any peak in protein, nor does there seem to be a significant amount of protein present in the active fractions. This suggests that conusine is not a protein. To further investigate this possibility, the bioactive G-50 fractions were pooled, lyophilized, and tested for stability in a number of experiments. It was found that neither heating at 100°C for 1 hr nor exposure to pH 2 or pH 10 detectably diminished the activity of G-50-purified conusine, but boiling 1 hr in 1.0 M HCl or 1.0 M NaOH did cause complete inactivation. Incubation with 10 mM DTT, a disulfide reducing agent of very low redox potential (13), had no effect on conusine activity, eliminating the possibility that conusine is a protein stabilized by intramolecular disulfide bonds. [Intramolecular disulfide bonds can stabilize proteins to a great degree, as evidenced by the 8000 MW peptide neurotoxin from the sea snake Laticauda semifasciata, which is stable from pH 1-11 and to heating at 100°C for 30 minutes (100). Bgt from the snake Bungarus multicinctus and cobrotoxins from Naja sp. also show considerable heat and pH stability (71). In all of these peptide toxins, it is the presence of intramolecular disulfide bonds which confers the stability, and reduction of the disulfide bonds denatures the toxins completely (100, 117).] In addition, none of the four proteases investigated--trypsin, chymotrypsin, pepsin, or pronase--detectably diminished the activity of conusine. From these results conusine was concluded to be non-protein. (DNAse and RNAse were also found to be ineffective in degrading the molecule.)

Once conusine had been clearly shown to be non-protein it was reasonable to try extracting it from the whole venom with solvents other than aqueous solutions. Methanol was found to extract virtually all conusine activity from the venom. Figure 6 shows the G-50 elution profile of methanol extracted venom. There is, as would be expected, virtually no protein extracted into the methanol, but the large peak in A_{280} which coincides roughly with the peak in bioactivity (referred to as G-50 III) is still present, as is the much smaller and rather broad peak immediately following it (G-50 IV). The A_{280} of the void volume peak is considerably reduced with respect to G-50 III.

The G-50 IV peak was shown by several methods--by TLC (Figure 7), by reaction with Ehrlich's reagent (an indole-reactive agent), and by its excitatory effect on the clam heart (see bioassays from "region C", i.e., G-50 IV, in Figure 5), to contain primarily 5-HT. This agrees with the conclusion of Cottrell and Twarog (18), based on TLC studies and the depolarizing effect of C. californicus venom on the serotonergic cells of the Helix buccal ganglion, that the venom contains 5-HT. This is not unusual, as 5-HT is quite commonly found in animal venoms (89).

Preliminary experiments investigating the batch-wise adsorption of conusine to various ion exchange resins were conducted in an attempt to determine an appropriate second step in the purification. Figure 8 illustrates and summarizes the results of these experiments. Conusine was clearly shown not to adsorb to the anion exchanger DEAE-Sephadex (in low salt and at pH 8.3) but its behavior with the

cation exchangers CM- and SP-Sephadex was more difficult to interpret. In the case of CM-Sephadex, most of the conusine activity appeared to adsorb to the resin in low salt and elute in high salt. However, there was a clearly detectable, lesser amount of activity which did not adsorb in low salt. SP-Sephadex, a stronger cation exchanger, was tried in an attempt to obtain tighter adsorption of conusine to the resin in low salt, but in this case, even less conusine appeared to adsorb to the resin. The low and high salt washes of the SP-Sephadex appeared to contain approximately equal amounts of conusine bioactivity. It was concluded, therefore, that Sephadex ion exchange resins were inappropriate for the purification of conusine.

Analytical TLC of G-50-purified conusine on silica gel plates indicated that a number of components could be resolved with this technique (Figure 9). This method was therefore scaled up to "semi-preparative" proportions on 0.5 mm silica gel plates, in order to run sufficient G-50 III to allow bioassay of the various regions of the chromatogram. It was found that on semi-preparative silica gel TLCs, in the two solvent systems ethanol-ammonium hydroxide-water, 7:2:1, and ethanol-water, 7:3, conusine activity remained at the origin (Figure 10). Nevertheless, because this step appeared to remove a number of components, it was adopted as a second stage in the purification of conusine.

One of the components removed by the semi-preparative TLC step is evidently responsible for the large A_{280} peak with which conusine activity coincides in the G-50 elution profile, since silica gel TLC-purified conusine (sg TLC 1) does not show any absorption in the UV

region. This fact was dramatically demonstrated in several experiments in which silica gel TLC-purified conusine was desalted on either G-10 or G-15 Sephadex columns. Figure 11 shows that, in one such desalting run on a calibrated G-10 column, although there is virtually no absorption at 280 nm in the elution profile, there is a very sharp peak in activity in the region corresponding to a molecular weight between that of ATP and 5'AMP (around 450 daltons). (From the calibration standards which were run on the G-50 column, it is clear that conusine elutes beyond the calibratable fractionation region of this resin. Thus, the elution position of conusine on G-50 cannot be used to obtain a reliable molecular weight estimate of the molecule. G-50 remains a more suitable resin than one with a smaller pore size for the initial purification step, however, since it resolves the very high molecular weight peak (G-50 I) and G-50 IV from G-50 III much better than does even G-25 Sephadex.)

Analytical cellulose TLC of silica gel TLC-purified conusine indicates that the extraction procedure used to elute the conusine from the silica gel also extracts significant amounts of the organic binder used in the preparation of the silica gel plates (Figure 12). A third purification step was judged necessary to remove the binder, as well as to resolve what appeared on the cellulose TLCs to be two components in the sg TLC 1 sample.

Thin layer electrophoresis (TLE) was investigated as a possible final step in the purification procedure. Analytical TLE of conusine at a number of pHs from 6.5 (Figure 13) to 11.0 indicated that conusine

was positively charged even up to pH 11.0 and showed a higher electrophoretic mobility than ACh (Figure 13). At all pH's, G-50-purified conusine and silica gel TLC-purified conusine ran with about the same electrophoretic mobility (with slightly greater tailing in the case of G-50 III) and as a single detectable spot (indicating that TLE on cellulose does not provide as good a resolution, of the components in G-50-III at least, as does TLC on silica gel). A semi-preparative TLE on a 0.5 mm cellulose sheet confirmed that the single detectable spot (by iodine vapor staining) contained the conusine activity, but recoveries were very poor.

Since silica gel TLC-purified conusine was shown to be resolved into at least two components on analytical cellulose TLC, a semi-preparative TLC on cellulose was chosen as the third purification step. Figure 14 shows that semi-preparative cellulose TLC of sg TLC 1 gave only one major band, with an R_f of $\sim$ 0.72, and a second, faint band at the origin, which was the organic binder from the silica gel TLC. Unfortunately, aqueous extraction of the major band, which contained the conusine activity (see Figure 14), also extracted several contaminants from the cellulose, as shown by analytical cellulose TLC of the cellulose TLC-purified conusine (cell TLC 4) and of aqueous extracts of blank cellulose plates (Figure 15). A comparison of sg TLC 1 and cell TLC 4 on the analytical cellulose TLCs in Figure 15 reveals that the semi-preparative cellulose TLC step has indeed separated the lesser of the two components of sg TLC 1 from conusine, even though this component was not detectable as a band on the semi-preparative cellulose TLC.

Both the physiological activity of conusine (see part 2 of Results, below) and its chemical properties suggest the presence of one or more quaternary nitrogens in the molecule. Drugs which are capable of activating or blocking a cholinergic receptor generally contain one or more quaternary nitrogens. From the results of the ion exchanger and TLE experiments, it is clear that conusine is positively charged, and the most likely function to confer this charge is a quaternary nitrogen. Therefore, conusine was compared to a number of quaternary ammonium compounds by paper chromatography and analytical TLC. Table I summarizes the results of a number of such chromatograms. The primary conclusion which can be drawn from such comparisons is that conusine is extremely polar, i.e., it almost certainly does not contain any aromatic groups and must possess polar functional groups over and above quaternary nitrogens, since it shows lower R_f values than any of the other quaternary ammonium compounds.

The reaction of various spray reagents with purified conusine on TLCs gives further information as to what functional groups are present and what groups are probably absent in the sample. In all of the analytical TLCs used in spray reagent tests, the quantity of conusine loaded was sufficient to give a spot clearly detectable by iodine vapor staining, and in most cases was equal to 60 μg or more of sample material. Conusine did not test positive with ninhydrin or Ehrlich's reagent. These results indicate the absence of primary amino functions or indole groups, respectively. Of the reagents generally used to detect quaternary ammonium compounds, only dipicrylamine and the Theis-Rutherford modification of Dragendorf's reagent gave a positive color reaction with conusine. However, although the remaining quaternary nitrogen-active reagents

which were tried (iodoplatinate, the Munier modification of Dragendorf's reagent, and the Munier-Macheboef modification of Dragendorf's reagent) did not produce positive color reactions, the conusine spot was visible as a white or pale spot against the background color. Such quenching may well be due to a specific reaction, and is often taken to indicate a positive test. These results are, in general, consistent with and indicative of the presence of a quaternary nitrogen function. Since the specificity of a given reagent is often empirical, and the exact molecular requirements for the chemical reaction which gives rise to the color are usually not known, it is impossible to determine why conusine should give the color reaction with some quaternary nitrogen-active reagents and quench others. In the experiments in which these reagents were tested, identical aliquots of the same conusine stock solution were loaded on a series of TLC plates, developed, and each plate was sprayed with a different reagent.

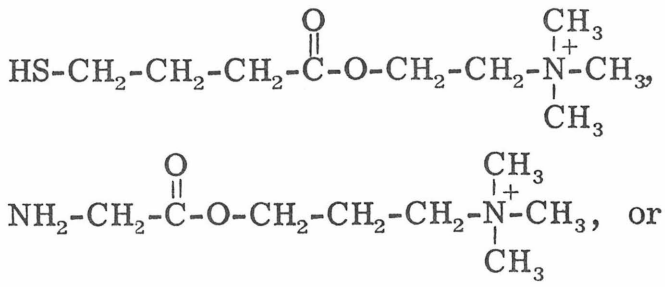
Phosphomolybdic acid, used to detect choline and choline esters, did not give a positive test with conusine, while hydroxamate-ferric chloride, a general carboxylic ester indicator, produced a pale spot against the background. Conusine was found to be completely stable to in vitro incubation with both AChE and BuChE, indicating either that it is not a choline ester, or that it must possess some function that confers considerable resistance to hydrolysis (such as the amide function of carbamylcholine, which is only very slowly hydrolyzed by AChE).

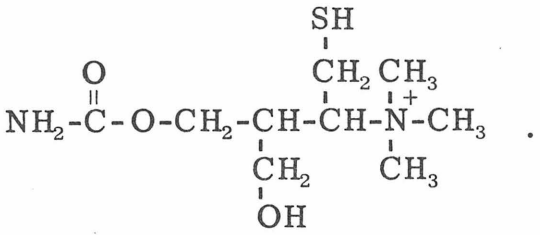
Figure 16 shows an IR spectrum of conusine purified through the cellulose TLC step. Although the sample used in this experiment contained conusine from 104 snails, there was clearly insufficient material present to obtain a very informative spectrum. The broad peaks centered at 3400 cm^{-1} and 1625 cm^{-1} are both due to water in the KBr pellet

(residual water left after lyophilization of the sample and/or water picked up from the atmosphere). The absorptions in the region of 1000-1200 cm^{-1} are not sufficiently resolved to be useful. Absorptions in this low energy region and lower are, moreover, of limited value in determining chemical structure since they generally are not very characteristic, i.e., reliably assignable to a particular type of bond. A faint suggestion of an absorption is present around 3100 cm^{-1}, in the region of C–H stretch absorptions. All organic compounds absorb here, and the small size of this peak indicates little material. The clear peak at 1680-1690 cm^{-1} occurs in the region of the major carbonyl absorption, which is generally the strongest peak in the IR spectrum of a carbonyl compound. The large sharp peak at 1390 cm^{-1} is very uncharacteristic of the rest of the spectrum, and could possible be due to some inorganic impurity. This peak occurs exactly at the position of the N–H bending absorption of the ammonium ion and is slightly higher than that of the C–N stretch of most tertiary and quaternary nitrogen compounds. The most likely interpretation of this spectrum is that the peak at 1680-1690 cm^{-1} is the strongest absorption of the conusine spectrum and indicates the presence of a carbonyl function, while the rest of the absorptions attributable to functional groups of conusine are not detectable, due to low concentration of the sample.

The Fourier transform NMR ^1H spectrum of cellulose TLC-purified conusine in D_2O (obtained with the help of Dr. Ken Reed) is shown in Figure 17. Most of the absorptions are barely resolved from the background noise, indicating again that the concentration of conusine in the sample, although high in terms of bioactivity (104 venom ducts/0.5 ml) is still very low in terms of the chemical

concentration. The large absorption which is numbered 3 in Figure 17 can be assigned with a great deal of certainty to methyl protons on a quaternary nitrogen. The τ value of this peak, 7.05, corresponds exactly with the τ value generally assigned to such protons, and the absorptions arising from methyl protons on the quaternary nitrogens of carbamylcholine, decamethonium, and other similar compounds have been shown to occur at this frequency in D_2O. Assignments of the remaining absorptions are less certain, largely due to their poor resolution from background. The complementary information provided by splitting of the peaks due to coupling between protons on adjacent atoms and by the relative areas under the peaks is lacking in this spectrum. However, some likely assignments can be made and are listed in the legend to Figure 17. The absorptions present are consistent with, though not necessarily indicative of, the presence of methylene groups adjacent to a hydroxyl, methylenes next to a sulfhydryl, a methylene adjacent to an esteratic function, and possibly a methylene adjacent to a tertiary nitrogen. Exchangable protons, i.e., hydroxyl, sulfhydryl, and amino protons, would not be directly detectable in this spectrum, since they exchange readily with the protons of D_2O. Some possible structures which could accommodate the information obtainable from this spectrum are:





These hypothetical structures are offered as guides to the general type of molecule which is indicated by the NMR data.

Attempts were made (in collaboration with Dr. D. J. Jenden) to obtain a mass spectrum of conusine using the procedure of Jenden et al. (52, 53) for N-demethylation of choline compounds to give a volatile derivative which is first purified and characterized by gas chromatography and then introduced into a mass spectrometer. In preliminary experiments, the conusine sample (silica gel TLC-purified conusine from 25 Conus) contained barely enough material to detect. The mass spectrum showed a small peak at a mass/charge ratio of 58, which corresponds to that of the $(CH_3)_2N^+=CH_2$ ion, which is the base peak for choline esters and related compounds (52). Behavior on the gas chromatograph was consistent with the presence of a carbonyl function. Surprisingly, conusine showed a retention time which was almost exactly that of ACh. The results from these preliminary experiments are consistent with the other spectral data on conusine. Further studies, using greater quantities of conusine, should provide more definitive information on the chemical structure of conusine.

2. Physiological Activity of Conusine

Conusine was found to have a marked inhibitory effect on the in vitro spontaneously beating heart of the clam Mercenaria mercenaria. As shown in Figure 18, addition of conusine to the bath was followed by a decrease in amplitude and frequency of the spontaneous beat. The degree of decrease in amplitude and frequency, the time between application of conusine to bath and onset of the inhibition, and the time required for recovery of the normal beat, were all functions of the concentration of conusine added. At sufficiently high concentrations, the heart was arrested in diastole immediately upon application of conusine and recovery did not occur for several hours after rinsing in ASW. Recovery could generally be speeded by addition of 5-HT (sufficient to give a final concentration of 10^{-7} g/ml or 2.6×10^{-7} M) to the bath. It is of interest to note in Figure 18 that contraction of the heart can be induced by mechanical means (such as, in this case, allowing the bath to run dry) during conusine inhibition of the spontaneous beat. This indicates that conusine is not interfering with muscle contraction per se.

The conusine used in the physiology experiments described below had been purified past the first or second step of the standard purification procedure, i.e., G-50 fractionation on silica gel TLC. The absolute concentration in grams/liter of conusine in a given sample could not be determined, since conusine was never obtained in a form sufficiently free from impurities (generally, contaminants from the "purification" procedure, such as ammonium acetate or silica gel binder) to allow an accurate determination of the weight of conusine present. The concentrations of conusine used in the various experiments below are

given as the dilution of a stock conusine solution. Direct comparisons of such conusine dilutions can be made, then, only within and between experiments in which the same stock conusine solution was used.

One absolute measurement which could be made was the number of Conus, or the number of venom ducts, from which a conusine stock solution was derived. Using this measurement, the effective concentration of conusine on clam heart can be computed to be between $\sim 5 \times 10^{-2}$ ducts/ml and $\sim 5 \times 10^{-3}$ ducts/ml. The range in effective concentration arises from differences in the size of Conus specimens used and thus in the amount of venom obtained from a single snail, and from variations in the sensitivity of different clam hearts to the same concentration of a conusine sample.

As discussed in the Introduction, ACh also causes an inhibition of the spontaneously beating clam heart. Inhibition by a series of increasing concentrations of ACh is compared in Figure 18 with inhibition by increasing concentrations of conusine. The effects on the heart of the two agents can be seen to be very similar. Although ACh in sufficiently high concentrations also causes complete diastolic arrest, recovery from the complete arrest induced by conusine is generally much slower and more gradual than in the case of ACh, requiring more ASW rinses, more 5-HT additions, and a greater length of time.

The agents BZQ and TEA, which, as described in the Introduction, have been found to act as cholinergic antagonists on the clam heart (75, 109), were also found to block conusine inhibition (Figures 19 and 20). Both the threshold concentrations and the concentrations of antagonist required for total blockade were approximately

the same for antagonism of ACh and conusine. As has been reported (75), BZQ was found to be about 1000 times more effective an antagonist of both ACh and conusine action than was TEA. Greenberg (36) has noted that antagonism of cholinergic action in the clam heart by BZQ is only partially reversible, even after extensive rinsing. Similar irreversibility was observed in these experiments for BZQ antagonism of both ACh and conusine, as well as for antagonism by TEA. Extensive washing over periods of up to $1\frac{1}{2}$ hours did not restore full pre-antagonist sensitivity to ACh or conusine.

Several drugs which are effective cholinergic antagonists in vertebrate cholinergic systems were tested for antagonism of conusine action in the clam heart. dTC (Figure 21), hexamethonium, flaxedil, and atropine all had no effect on conusine inhibition, even at very high concentrations (10^{-3}M). These drugs have all been reported to be ineffective as cholinergic antagonists in the clam heart (75, 84, 109, 110) and this was verified here. [In these experiments, atropine often proved to be quite toxic to the heart, as Prosser (84) has reported, in concentrations of about 5×10^{-5} M and higher. At concentrations below this, and in most of the few instances when atropine did not abolish the spontaneous beating at these concentrations and higher, no blockade of conusine or ACh action was observed. In a single instance, atropine caused a marked excitation of the heart at concentrations of 10^{-5} M, 5×10^{-5} M and 10^{-4} M and simultaneously showed a progressive blockade of ACh action. This second type of activity by atropine appears to be similar to that reported by Welsh and Taub (110) who found atropine to be an effective cholinergic antagonist at 10^{-3} M. These investigators apparently observed

the same anomalous results with atropine on different hearts as were observed here, since in an earlier paper (109) they had reported no antagonism by atropine at any concentration.]

The cholinergic-like inhibition of the clam heart by conusine and its antagonism by agents which also block ACh action could be interpreted in one of three ways. (1) Conusine could be inhibiting the clam heart AChE, thus allowing endogenous ACh to act. (2) Conusine could be causing the release of ACh from pre-synaptic stores in the heart. (3) Conusine could itself be interacting with and activating a cholinergic receptor in the clam heart.

The first possibility assumes that endogeneous ACh is being continuously released or leaked and that the AChE present normally degrades it and prevents it from acting postsynaptically. Such a hypothetical mechanism is unlikely, in view of the fact that known anticholinesterase agents, such as eserine, do not exhibit a direct inhibitory effect on the heart (84, 105, 109). Nevertheless, the effect of conusine on AChE activity was investigated in order to definitely exclude this mechanism. As Figure 22 shows, conusine exhibits no inhibition of AChE from either Torpedo electroplax or clam heart. In fact, conusine appeared to enhance the in vitro activity of Torpedo AChE. This phenomenon was neither explained nor further investigated, except to demonstrate that conusine alone shows no esterase activity in the spectrophotometric assay. It can be noted from Figure 22 that the AChE activity present in clam heart is quite low, as has been reported by others (54, 87, 94), but is clearly detectable and is completely inhibited by 10^{-4} M eserine and neostigmine.

The second possible mechanism of conusine action on the clam heart, release of endogenous ACh, was investigated by attempting to

determine whether inhibition by conusine could be distinguished from inhibition by ACh, by means of different sensitivities to the anti-cholinesterase activity of eserine. As reported in part 1 of the Results, both AChE and BuChE were shown to be totally without effect on conusine. Thus, it was assumed that conusine was not degraded by the endogenous AChE of clam heart. If eserine were to potentiate the effect of conusine, then, it would indicate that the conusine action is mediated by ACh, which is sensitive to AChE activity and should be potentiated by AChE inhibitors. If, on the other hand, eserine could be shown to potentiate ACh action but not conusine action, it would indicate that the mechanism of inhibition by conusine does not involve the release of ACh.

Some difficulty could be anticipated in obtaining clear results from such an experiment, due to the very low levels of AChE present in clam heart. Prosser (84) has reported that 10^{-4} g/ml ($\sim 4 \times 10^{-3}$ M) eserine only slightly potentiates the action of ACh on Mercenaria heart, and Welsh and Taub (109) have estimated such potentiation to be 2 to 5 fold. However, in the experiments which were performed here to compare the effect of eserine on conusine action and on ACh action, no potentiation of even ACh action was observed (Figure 23). The cholinergic blocking action of eserine apparently predominated over any effects due to inhibition of esterase activity, even at concentrations as low as 5×10^{-6} M. Eserine also blocked conusine action at these low concentrations.

Failure to observe potentiation of ACh inhibition by eserine may have been due to an insufficient incubation period in eserine prior to

ACh application. Prosser (84) described an incubation period of 15-20 minutes for eserine potentiation, and in the experiments reported here, hearts were bathed in eserine for only 5 minutes before testing with ACh and conusine. Also, it may have been erroneous to assume, as is the case in vertebrate preparations (62), that the anti-cholinesterase activity of eserine in this system should be expressed first, i.e., at lowest concentrations, and the receptor blocking activity should be expressed only at higher concentrations. Thus it may be that the experiments were discontinued prematurely and higher concentrations of eserine should have been tested. (It is of interest to note that the observation of eserine blockade of ACh action is in contradiction to the report by Luduena and Brown (75) that 2×10^{-4} M eserine produced no cholinergic antagonism in Mercenaria heart.)

Prosser described the major effect of eserine to be a prolongation of the inhibition of ACh added to the bath. The relatively longer time required for recovery from conusine as compared to recovery from ACh suggests that conusine-induced inhibition may be less sensitive to endogenous AChE than is ACh inhibition, and thus may be taken to provide a bit of indirect evidence regarding the site of action of conusine. Another approach to distinguishing the second and third mechanisms of conusine inhibition, which was not tried but might give more definitive results than experiments with eserine, would be to compare the effect of AChE added to the bath on ACh inhibition with the effect of AChE on conusine inhibition.

Evidence which excludes the second mechanism of conusine inhibition and at the same time proves the third, was obtained not in

the clam heart but in another molluscan system, the Aplysia pleural ganglion. The pharmacology of the cholinergic response in the clam heart, in particular, antagonism by BZQ and TEA and the lack of antagonism by dTC, atropine or other classical cholinergic drugs, suggested that the cholinergic receptor of the heart might be similar to the unique cholinergic receptor which mediates a slow hyperpolarization in Aplysia neurons, as described by Kehoe (57, 58, 59). If these two receptors are, indeed, of the same type, then Aplysia neurons should provide an ideal system in which to test the action of conusine using the very definitive techniques of intracellular recording and iontophoretic drug application. In addition, since there are three different types of cholinergic receptor present on the various neurons in the Aplysia pleural ganglion, this ganglion carries the potential of providing a system in which the action of conusine can be discriminated from that of ACh. If conusine itself directly activates the cholinergic receptor in clam heart, it might be predicted that conusine should evoke a slow hyperpolarizing response when iontophoresed onto the medial cells of the pleural ganglion of Aplysia, which cells have been shown to bear the cholinergic receptor mediating the slow hyperpolarizing, K^+-dependent response. If, in addition, conusine is more specific in its interaction with this type of receptor than is ACh, then conusine would not activate the other two types of cholinergic receptors which are also found in cells of the pleural ganglion. These possibilities were investigated, with the help of Dr. JacSue Kehoe, in a series of experiments on the Aplysia pleural ganglion.

The effect of conusine iontophoresed onto the soma of a medial cell of the Aplysia pleural ganglion at several preset membrane potentials is shown in Figure 24, along with the response (of a different medial cell) to iontophoretically applied ACh at the same potentials. The two phases, rapid and slow, of the hyperpolarization elicited by ACh can be distinguished by the disappearance of each phase at its respective inversion potential. As described in the Introduction, Kehoe has demonstrated that an increase in Cl^- conductance underlies the early, rapid hyperpolarization which inverts at -60 mV (the Cl^- equilibrium potential in this system), whereas an increase in K^+ conductance underlies the slow hyperpolarization which inverts at -80 mV (the K^+ equilibrium potential). In contrast to the action of ACh, conusine elicits only the slow component of the response. This action of conusine was blocked by 10^{-3} g/ml MXC (Figure 25) and by 10^{-3} g/ml TEA (Figure 26), both of which selectively block the slow hyperpolarizing response. The action of conusine was not blocked by dTC, which blocks the other two types of cholinergic response in Aplysia neurons.

The AChE inhibitor neostigmine, which prolongs the action of ACh on the medial cells (J. S. Kehoe, personal communication), did not prolong or enhance the response to conusine (Figure 27). On the contrary, at the concentration used it antagonized the conusine response to some extent. This is not surprising, since AChE inhibitors, as mentioned above, generally exhibit additional cholinergic effects, including receptor blockade (62). This finding does show that conusine

is not hydrolyzed by endogenous esterases, confirming the results obtained in the experiments in which conusine was incubated with AChE and BuChE.

It is of interest to note that the time course of action of conusine applied iontophoretically onto the medial cells is approximately the same as that of ACh, i.e., the hyperpolarization following a brief pulse is transient, presumably due to a diffusion of conusine away from the receptors. Bath application of conusine resulted in a prolonged hyperpolarization of the medial cells (Figure 28).

I-V curves obtained from the medial cells both before and during conusine bath application showed that it, like ACh, causes a marked increase in membrane conductance.

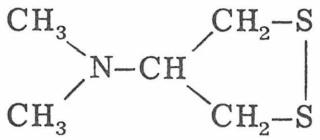
Neither iontophoretically applied nor bath applied conusine had any effect on the anterior cells of the pleural ganglion (Figure 28), which bear only the receptors mediating a depolarizing response (caused by an increase in Na^+ conductance) which is blocked by dTC and hexamethonium. Likewise, bath applied conusine had no effect on IPN I (the cholinergic cell which synapses on both the medial and the anterior cells), which shows only the rapid, Cl^--dependent hyperpolarization in response to ACh. These results confirm that conusine is a specific agonist of the cholinergic receptor mediating the slow K^+-dependent response in Aplysia neurons, and support the conclusion that conusine is acting in the clam heart by direct activation of the cholinergic receptor in that system as well.

The above experiments on Aplysia neurons and Mercenaria cardiac tissue clearly show conusine to be a neuroactive agent in invertebrates. In an attempt to investigate possible effects of conusine on a vertebrate system, conusine was assayed on the frog rectus abdominis and on the isolated, perfused frog heart. In neither of these systems did conusine exhibit a cholinergic agonistic effect at concentrations of approximately 10 times that which were effective on the clam heart, nor did conusine appear to antagonize the effect of ACh on the rectus abdominis (Figure 29).

As a footnote to the discussion of conusine's physiological effects, it may be of interest to report the results of experiments which were performed early in the investigation of conusine's action on the clam heart, at which time the clam heart Na^+-K^+ ATPase was considered a possible site of conusine action. In these experiments (performed with the help of Dr. Juta Reed) conusine was incubated with membrane-bound ATPase from eel electroplax and the ATPase activity was assayed in an in vitro spectrophotometric assay. Conusine was found to have no effect on ATPase activity.

In an exploratory experiment, it was observed that very high concentrations of nereistoxin (NTX), a neurotoxin from the marine polychaete worm Lumbriconereis heteropoda, blocked the action of conusine on clam heart but had no effect on the action of ACh (Figure 30). NTX has been shown to act as a cholinergic antagonist on the ACh receptor of the vertebrate neuromuscular junction (79) and in spinal motor neurons as well (12). However, its antagonism of

conusine action may not be related to this property, since such high concentrations of NTX are required and since NTX does not antagonize the action of ACh on the heart. It may be that the source of NTX antagonism of conusine action is the disulfide bond in NTX:



It has been suggested by Nagawa et al. (79) that NTX blocks the ACh receptor in the vertebrate neuromuscular junction by forming a disulfide bond with putative sulfhydryls on the receptor which are close to the ACh binding site or are otherwise essential to the activation of the receptor (see Karlin, 56, for review). It may be that NTX is interacting in a similar manner with some aspect of the ACh receptor in the clam heart which is not essential for ACh activation of the receptor but which is involved in conusine's interaction. (It is interesting to note that both in the case of batrachotoxin (1) and the Conus toxin from C. magus (101), a number of sulfhydryl-active reagents have been shown to antagonize the effects of the respective toxins, presumably by acting with sulfhydryl sites on the postsynaptic membrane which are essential to the action of the toxins.) Alternatively, NTX may be interacting directly through sulfhydryl bonding with the conusine molecule. Although the experiments with DTT indicate that conusine does not have any disulfide bonds which are necessary for activity, the presence of free sulfhydryl groups has not been excluded and is, in fact, consistent with the NMR data. A direct interaction between NTX and

conusine would explain why the effects of NTX are so immediately and completely reversible upon washing, as well as why they appear to be specific for conusine. Further experiments, investigating the effect of sulfhydryl-active reagents on conusine activity, may contribute to the elucidation either of the molecular mechanism of conusine interaction with the cholinergic receptor involved or of the structure of conusine itself.

3. Cholinergic Receptors in the Clam Heart

The previously described (see Introduction) pharmacological properties of the cholinergic response in Mercenaria heart and the action of conusine on both clam heart and the medial cells of the Aplysia pleural ganglion, indicate a strong similarity in the cholinergic receptor types of the two systems. This resemblance was investigated further by assaying on the clam heart some of those agonists and antagonists whose action had been characterized by Kehoe in Aplysia neurons but which had not been tried on clam heart.

Arecoline, the only agent (other than conusine) which has been found by Kehoe to specifically activate the cholinergic receptor which gives the slow hyperpolarizing response in Aplysia neurons, was found to be a very effective inhibitor of the clam heart, active in concentrations as low as 10^{-8} M in some hearts (Figure 31). MXC and PTMA, which have been described by Kehoe as specific antagonists of the slow hyperpolarizing cholinergic response in Aplysia neurons, also antagonized the action of ACh and conusine in clam heart (Figures 32 and 33). MXC was a much more effective antagonist than PTMA, and was

capable of complete blockade at concentrations of 10^{-7} to 5×10^{-7} M in some hearts. PTMA showed some antagonism at 10^{-6} M in some hearts, but completely blocked ACh or conusine at much higher concentrations--generally from 5×10^{-5} M to 5×10^{-4} M.

Table II lists these results along with the pharmacological results of other investigators for clam heart and of Kehoe for the unique receptor in Aplysia. Both the antagonists and agonists which are very effective (i.e., totally effective at low concentrations) in the clam heart are, but for two exceptions, either absolutely specific or relatively specific for the cholinergic receptor in Aplysia which mediates the slow hyperpolarizing response (i.e., they either do not act on the other two types of cholinergic receptor or they act only at much higher concentrations). Carbamylcholine and BZQ are somewhat exceptions to this correlation. While carbamylcholine is an effective cholinergic agonist in the clam heart at very low concentrations, it non-selectively activates all three cholinergic receptors in Aplysia neurons (57). Similarly, BZQ is an effective antagonist in the clam heart at very low concentrations, but blocks all three types of cholinergic response in Aplysia (Kehoe, personal communication). These two exceptions, however, are not inconsistent with the identity of the clam heart cholinergic receptor and the unique Aplysia cholinergic receptor. All of those agonists and antagonists which are totally without effect on the clam heart are also totally without effect on the Aplysia medial cell receptor. There are several agonists (nicotine, muscarine, methacholine, TMA) which are without effect on the Aplysia receptor but

which do act on the clam heart. These agonists characteristically act at much higher concentrations in the clam heart than those agonists which are effective on both receptors.

An additional cholinergic antagonist which was investigated on the clam heart was α-bungarotoxin (Bgt), a very effective and virtually irreversible antagonist of the ACh receptor in the vertebrate neuromuscular junction. Bgt was without effect on either conusine or ACh action in the clam heart at concentrations (10^{-6} M) which completely block ACh action at the neuromuscular junction. At much higher concentrations, 10^{-4} to 5×10^{-4} M, Bgt still showed no antagonism of conusine action (Figure 34), but appeared to block ACh action on the heart (Figure 35). This antagonism was easily reversible with ASW rinsing, however, and appears to have been a very non-specific drug effect. Bgt has been reported by Shain et al. (93) to block all three types of cholinergic receptors in Aplysia neurons, but a number of investigators have been unable to repeat these results (5; H. Lester, personal communication; F. Strumwasser, personal communication). In particular, Kehoe has not found Bgt to block the slow hyperpolarizing cholinergic response in the Aplysia pleural ganglion cells (J. S. Kehoe, personal communication).

An attempt was made to determine the ionic basis underlying cholinergic inhibition in Mercenaria heart by altering the external ionic composition of the ASW in the bath and looking for effects on the response to ACh. K^+ and Cl^- ions are the two major candidates for mediation of inhibition of a nerve or muscle cell. Increases in

permeability to K^+ (38, 44, 57, 98, 99), to Cl^- (6, 10, 57, 72, 82, 90), and to both K^+ and Cl^- (see 20 for review) have been shown to be responsible for inhibition in various vertebrate and invertebrate systems. [In the last case, increases in permeability to both K^+ and Cl^-, it has not been established whether inhibition results from activation of a single receptor coupled to both ionophores, or from activation of two receptors each coupled to a specific ionophore, as has been shown by Kehoe to be the case in a number of Aplysia neurons (59).] Therefore, experiments were performed on the clam heart in which the K^+ and Cl^- concentrations of the external ASW were altered.

Figure 36 presents a typical set of results obtained in experiments in which the external K^+ concentration was altered. Although in a few instances ACh appeared to be slightly more effective in K^+-free solution and slightly less effective in $2 \times K^+$ or $3 \times K^+$ solution, these differences were generally small and were not consistent. In general, the results point to no clear effect of alterations in external K^+ on the ACh response.

The results from experiments in which Cl^--free ASW was substituted for normal ASW were much more consistent but very puzzling. In all three hearts investigated, the effectiveness of ACh inhibition was increased by a factor of about two in Cl^--free ASW (Figure 37). The effect was easily reversible by returning the bath solution to normal ASW. The interpretation of this effect of Cl^--free ASW on the ACh response is not immediately obvious, since it is exactly the opposite of the effect expected if a change in Cl^- conductance were involved in the ACh response. For a response to be hyperpolarizing (and thus

inhibitory), it must be mediated either by an outward flow of positive ions or an inward flow of negative ions. If the ACh-induced hyperpolarization in clam heart were mediated by an increase in permeability to Cl^- one would expect the response to be abolished, or greatly decreased, in a Cl^--free external solution. It appears that, in this system, alteration of the external Cl^- concentration has effects (possibly secondary effects on ionic gradients across the cardiac muscle cell membrane, or effects on the processes involved in the contraction mechanism) which are not understood.

In summary, the results of the above experiments involving alteration of ionic composition of ASW do not clearly point to a change in permeability to either K^+ or Cl^- as the ionic mechanism underlying ACh inhibition of the clam heart. This is, of course, not to say that neither of these ions is involved, but rather that the ionic dependence is still undetermined.

Figure 4. ASW extract of Conus venom on Sephadex G-50.

In the experiment represented in this figure, the venom ducts from 16 Conus (30 mm average length) were homogenized in 1.0 ml ASW and centrifuged at 12,000 x g for 10 min to pellet insoluble material. The pellet was washed once with 0.3 ml ASW and recentrifuged. The combined supernatants were loaded on a Sephadex G-50 column equilibrated with 0.1 M ammonium acetate, pH 8.3, and eluted with the same buffer. The resulting fractions were monitored for absorption at 280 nm, for protein by the method of Lowry, and for bioactivity on the clam heart. Bioactivity at dilutions of 1:100 and 1:400 on two different hearts is expressed in an "activity profile", in which the activity of a given fraction has been rated from - to ≣, according to the following rating system:

- \- Slight (<20%), temporary decrease in amplitude and/or frequency of spontaneous heart beat. Recovery occurring without rinsing.
- = Marked (20-50%) decrease in amplitude and frequency. ASW rinse generally required for recovery.
- ≡ Gradual decline in amplitude and frequency to diastolic arrest. Recovery generally occurring after one or two ASW rinses.
- ≡ Complete abolition of heart beat within 5 sec of addition of sample. Recovery generally requiring several ASW rinses.
- ≡ Immediate, complete abolition of heart beat. Recovery requiring 5-HT in addition to extensive rinsing.
- ≣ Immediate, complete abolition of beat. No recovery within time period of experiment.

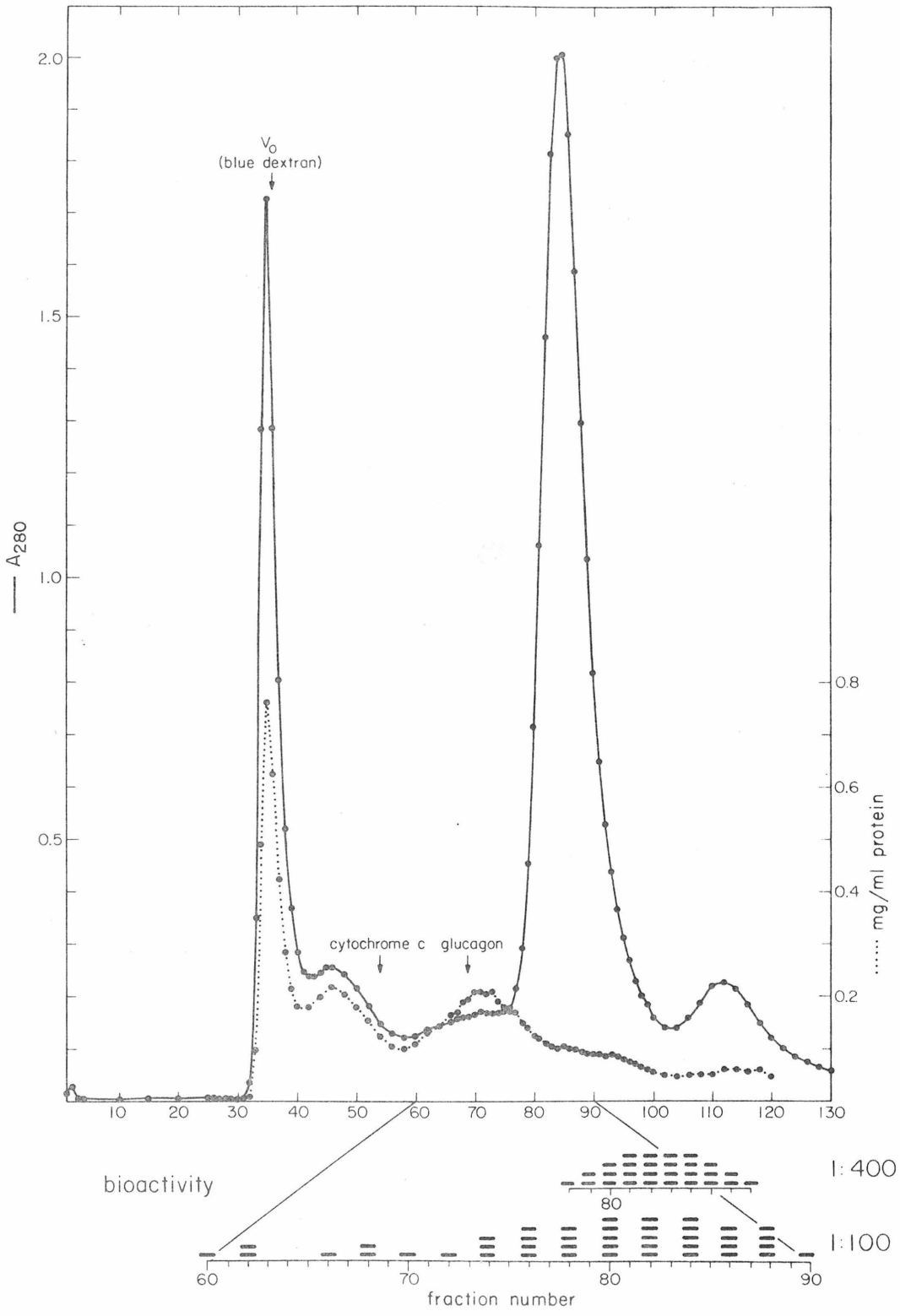

2.0
1.5
1.0
0.5
A_{280}
V_0
(blue dextran)
cytochrome c
glucagon
0.8
0.6
0.4
0.2
..... mg/ml protein
10
20
30
40
50
60
70
80
90
100
110
120
130
bioactivity
1:400
1:100
80
60
70
80
90
fraction number

Figure 4

Figure 5. Sample traces from activity profile of Conus venom fractionated on Sephadex G-50.

Traces showing the effect on clam heart of fractions of Conus venom from various regions in the G-50 elution profile are presented to illustrate the basis for the construction of activity profiles such as the one shown in Figure 4. The A_{280} profile which is presented is that of ASW-extracted venom on G-50. In each clam heart trace, the arrow marks the addition of a sample aliquot and the number above the arrow indicates the G-50 fraction from which that aliquot was taken. Fractions from regions I and II have no significant effect on the heart, while fractions from region III can be seen to exert a marked inhibition and fractions from region IV are excitatory. Dilutions in the traces corresponding to regions I+II and IV are from 1:20 to 1:40. Dilutions in the traces corresponding to region III are 1:100. Calibration: 30 sec, 0.1 g (I+II and IV); 12 sec, 0.1 g (III).

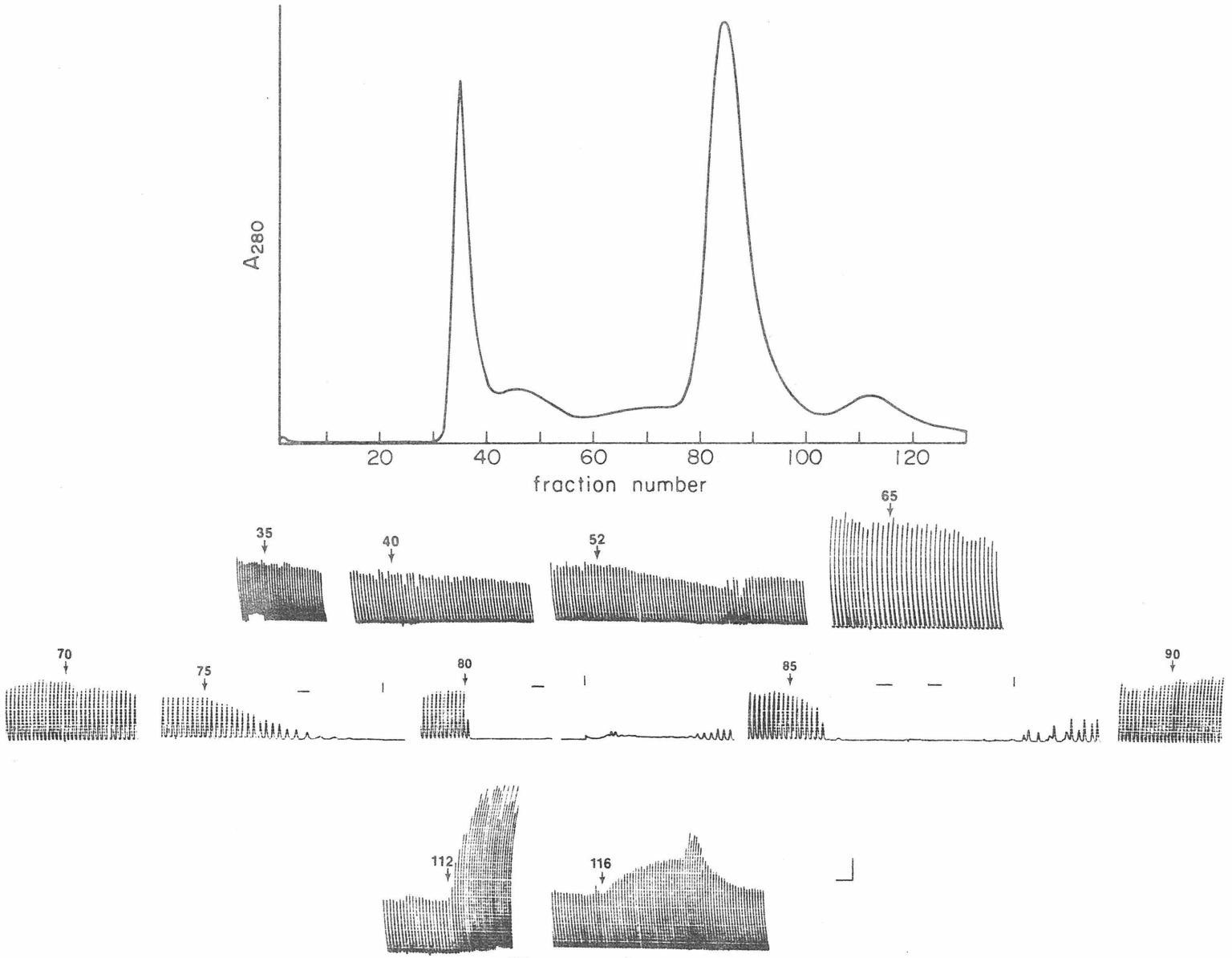

A280
20
40
60
80
100
120
fraction number
35
40
52
65
70
75
80
85
90
112
116

Figure 5

Figure 6. Methanol-soluble and methanol-insoluble extracts of Conus venom on Sephadex G-50.

In the experiment represented by this figure, venom ducts from 18 snails (29 mm average length) were homogenized in 4.0 ml methanol and centrifuged 15 min at 27, 000 × g to pellet insoluble material. The pellet was washed once with 2.0 ml methanol and the combined supernatants were evaporated, redissolved in 20 mM ammonium acetate, pH 8.0, and fractionated on a Sephadex G-50 column equilibrated in the same buffer. The methanol-insoluble pellet was resuspended and homogenized in 0.1 M ammonium acetate, pH 8.0, and spun at 27, 000 × g for 15 min. The pellet from this centrifugation was washed once in 0.1 M ammonium acetate and the combined supernatants were lyophilized, dissolved in 20 mM ammonium acetate, pH 8.0, and fractionated on G-50 Sephadex. G-50 fractions from both the methanol-soluble and methanol-insoluble extracts were monitored for absorption at 280 nm, for protein by the method of Lowry, and for bioactivity on the clam heart. Virtually all of the bioactivity was present in the fractions of the methanol-soluble extract.

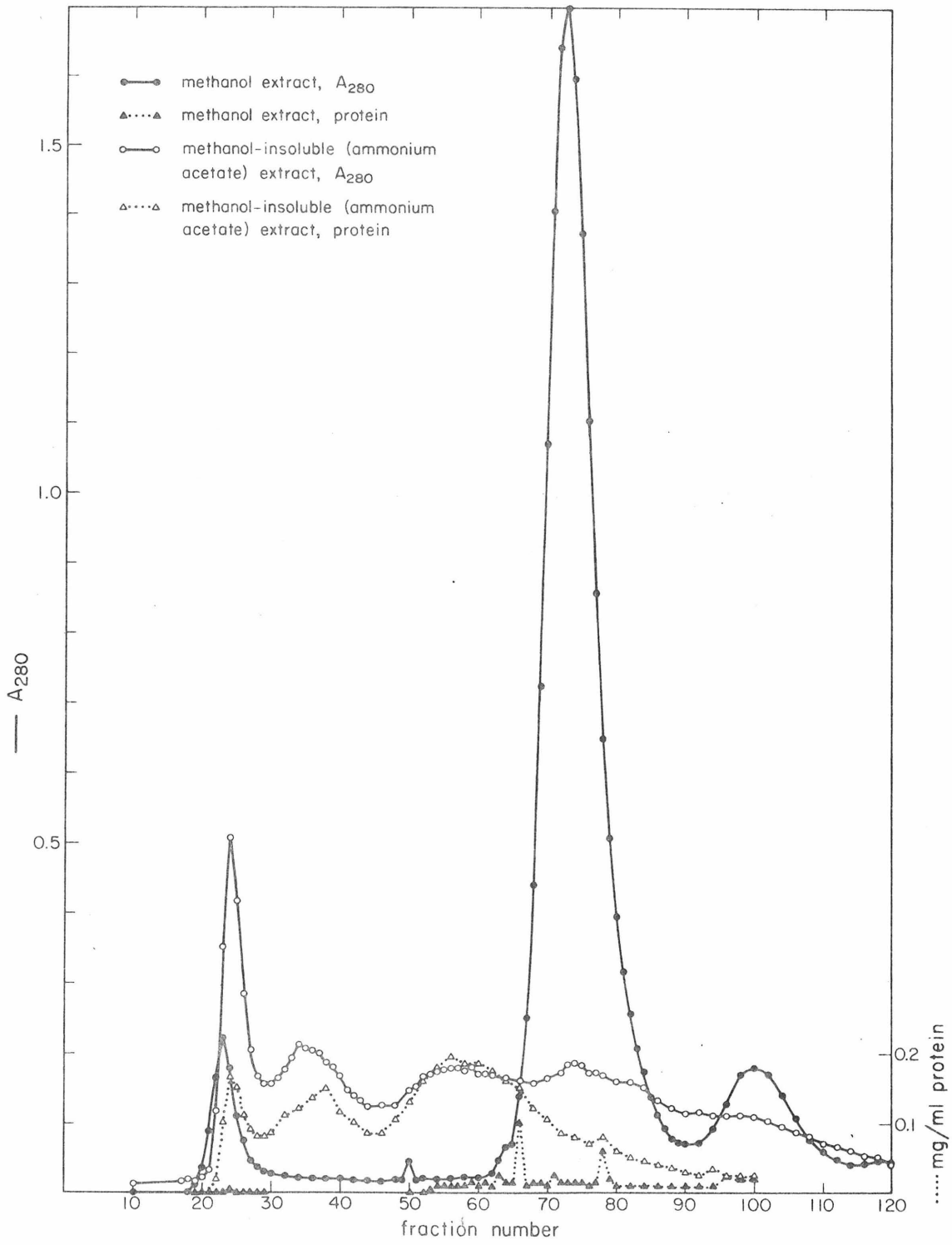

methanol extract, A_{280}
methanol extract, protein
methanol-insoluble (ammonium acetate) extract, A_{280}
methanol-insoluble (ammonium acetate) extract, protein
1.5
1.0
0.5
— A_{280}
0.2
0.1
····· mg/ml protein
10
20
30
40
50
60
70
80
90
100
110
120
fraction number

Figure 6

Figure 7. Analytical silica gel TLC of 5-HT and peak IV from G-50 fractionation of Conus venom.

The chromatograms show that the peak in A_{280} (G-50 IV) which follows the conusine-containing peak (G-50 III) in the A_{280} profile of Conus venom on Sephadex G-50, contains primarily 5-HT. 5-HT creatinine sulfate (1) and G-50 IV (2) were loaded onto three 0.25 mm silica gel plates and developed in one of the solvent systems, A, B, or C, described in Figure 9 and the Methods. The plates were then air dried and iodine vapor stained. The 5-HT creatinine sulfate used as a reference standard appears to contain several contaminants. The major spot of the 5-HT sample was taken to be 5-HT. The major spot in G-50 IV can be seen to co-chromatograph with the 5-HT spot. This G-50 IV spot also stains positively with Ehrlich's reagent, an indole-reactive reagent.

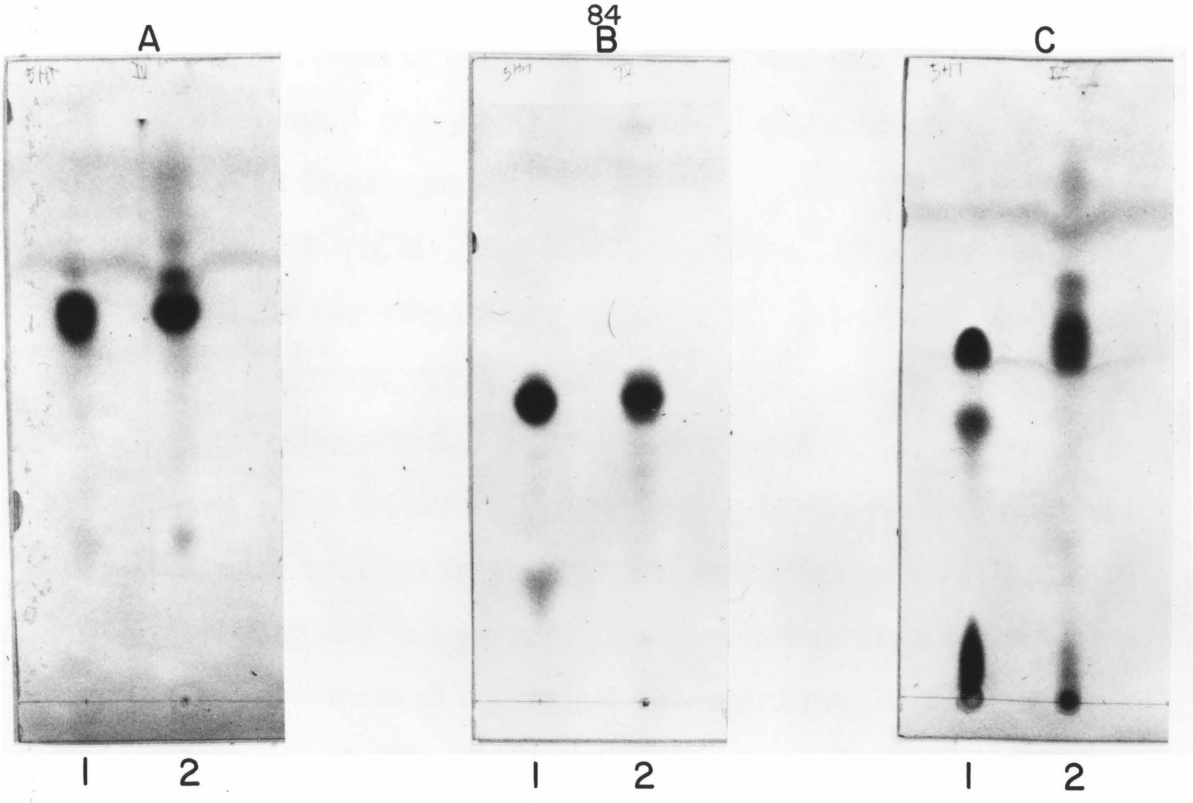
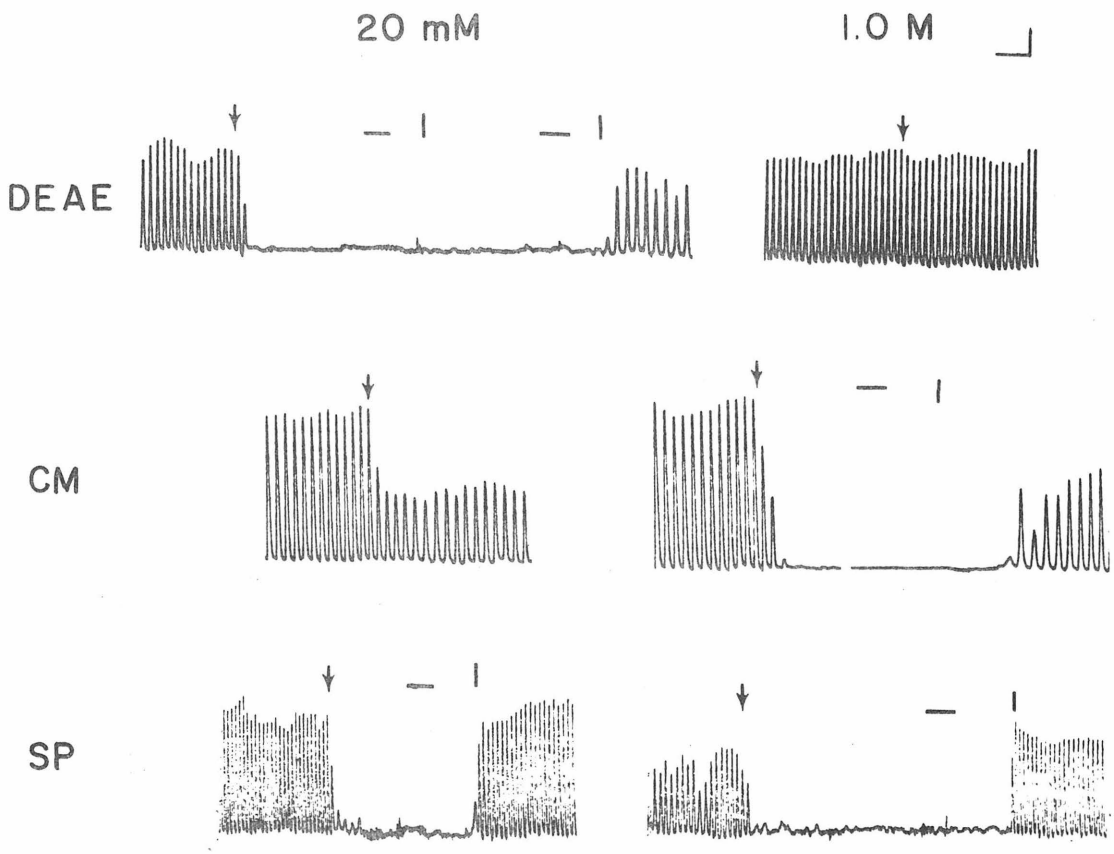


Figure 7

Figure 8. Adsorption of conusine to SP-, CM-, and DEAE-Sephadex.

Three equal conusine aliquots in 20 mM ammonium acetate were incubated (overnight with shaking) with one of the three ion exchange resins SP-, CM-, and DEAE-Sephadex. The slurries were pelleted and the supernatants, containing any conusine which was not adsorbed to the resin, were lyophilized and bioassayed. The pellets were then slurried with 1.0 M ammonium acetate, shaken overnight and pelleted. The supernatants, containing any conusine which had adsorbed to the resin in 20 mM salt but was released in 1.0 M salt, were lyophilized and bioassayed. Representative clam heart traces illustrating the effects of equivalent concentrations of the various supernatants are shown. Calibration: 30 sec, 0.1 g.

The results of bioassays of the various supernatants on a total of 14 hearts are summarized in the table shown. The supernatants from the incubation of conusine with DEAE-Sephadex and with SP-Sephadex were assayed on a total of 7 hearts each, and the supernatants from the incubation of conusine with CM-Sephadex were assayed on a total of 9 hearts. In the case of conusine incubated with DEAE-Sephadex, the 1.0 M supernatant showed no activity on any of the hearts assayed, while the 20 mM supernatant was fully active (i.e., as active as the untreated, control conusine sample) on all the hearts. On the other hand, in the cases of the conusine aliquots incubated with CM-Sephadex and SP-Sephadex, activity was found in both the 20 mM and the 1.0 M supernatants for all hearts tested. In the case of CM-incubated conusine, the 20 mM supernatant appeared to contain about twice the activity of the 1.0 M supernatant, while in the case of the SP-incubated conusine, both supernatants showed approximately the same activity.



	activity present in 20 mM wash	activity present in 1.0M wash	activity of 20 mM >1.0 M	activity of 20 mM=1.0M	activity of 20 mM<1.0 M
DEAE-Sephadex + conusine	7	0	7	0	0
CM-Sephadex + conusine	9	9	0	0	9
SP-Sephadex + conusine	7	7	0	7	0

Figure 8

Figure 9. Analytical silica gel TLC of conusine: whole venon, G-50 III and sg TLC 1.

(a) Aliquots of (1) whole Conus venom (methanol-extracted), (2) G-50 purified conusine (G-50 III) and (3) the G-50 IV peak (the peak in A_{280} which follows the activity-containing peak in the G-50 A_{280} profile) were loaded onto four 0.25 mm silica gel plates and developed in one of the following solvent systems:

A ethanol-ammonium hydroxide-water, 7:2:1

B butanol-acetic acid-water, 4:1:1

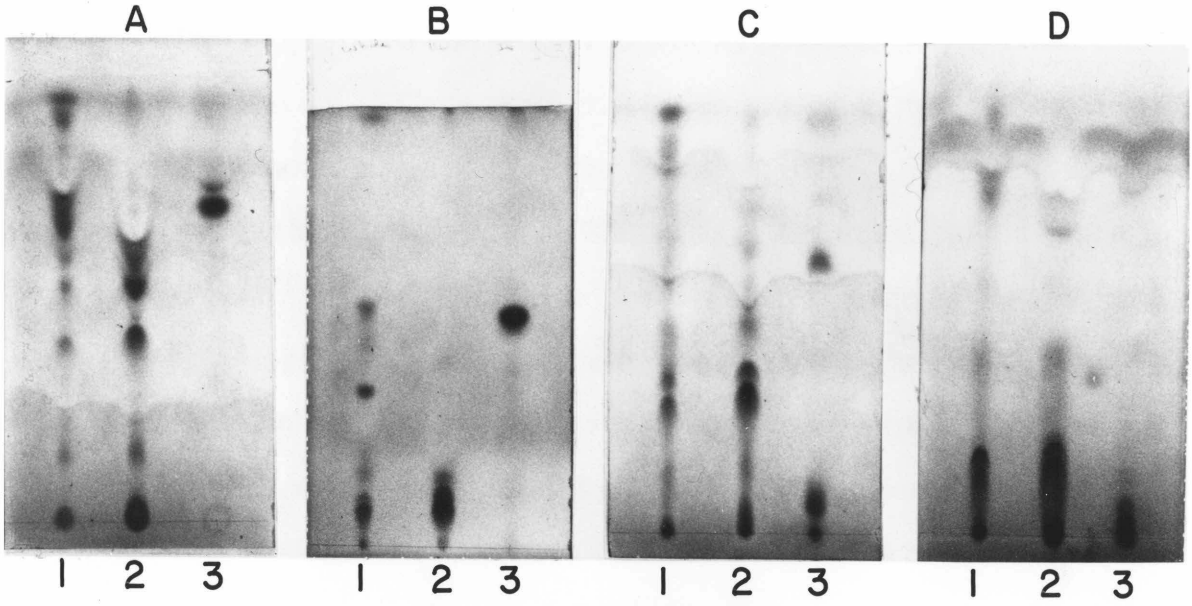
C ethanol-water, 7:3

D methanol

The plates were air dried and stained in an iodine vapor tank. The chromatograms show that there are a number of components present in the conusine-containing G-50 fraction, G-50 III.

(b) The chromatogram shows that a number of the components in G-50 III are eliminated by semi-preparative TLC on silica gel. The analytical silica gel plate shown was loaded with ACh, G-50 III, and conusine purified by TLC on 0.5 mm silica gel (sg TLC 1) in ethanol-ammonium hydroxide-water, 7:2:1. The analytical plate was then developed in ethanol-ammonium hydroxide-water, 7:2:1, air dried and iodine vapor stained.

(a)



(b)

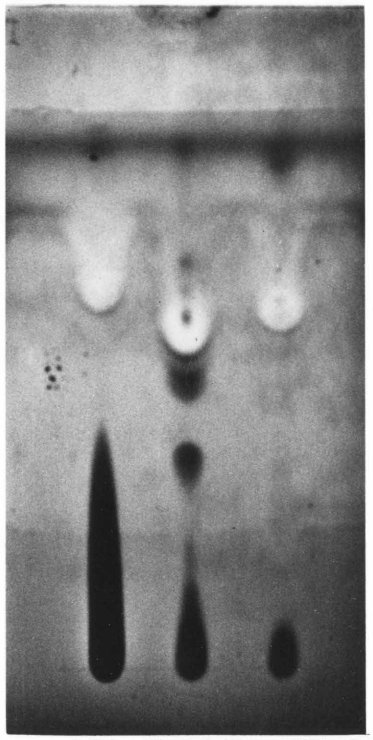


Figure 9

Figure 10. Semi-preparative silica gel TLC of G-50-purified conusine.

The lyophilized conusine-containing peak (G-50 III) from the G-50 fractionation of Conus venom was dissolved in methanol and loaded, by repeated spotting, on a 0.5 mm silica gel plate. The plate was developed in ethanol-water, 7:3, for $3\frac{1}{2}$ hrs, air dried, and stained in an iodine vapor tank. The center photo shows the entire plate at high contrast, to highlight the minor components of the chromatogram. The high-contrast photo on the right, taken at a lower exposure, highlights the minor components even further. The photo on the left, taken with normal contrast film, illustrates that the component at the origin is clearly the major component of G-50 III. The center and right photos represent the TLC of an extract from 41 Conus, 25 mm average length (approximately 15 mg of lyophilized G-50 III material). The left photo is a TLC of an extract from 53 Conus, 25.5 mm average length (21 mg of lyophilized G-50 III material).

The clam heart traces to the right demonstrate that the conusine bioactivity is located in the component at the origin. The various bands of the TLC were scraped from the plate and eluted with 1.0 M ammonium acetate, lyophilized, redissolved in 400 μl ASW, and bioassayed on a number of hearts at several dilutions. The traces show the activity on a single heart of 1:100 dilutions of the extracts from the various regions of the chromatogram. The arrows in the traces mark the addition of an aliquot from the indicated region of the TLC. In this particular assay, a little more than $\frac{1}{4}$ of the G-50-purified conusine from 16 Conus, 25.5 mm average length (approximately 1.8 mg of material), was loaded on the silica gel TLC. Calibration: 30 sec, 0.1 g.

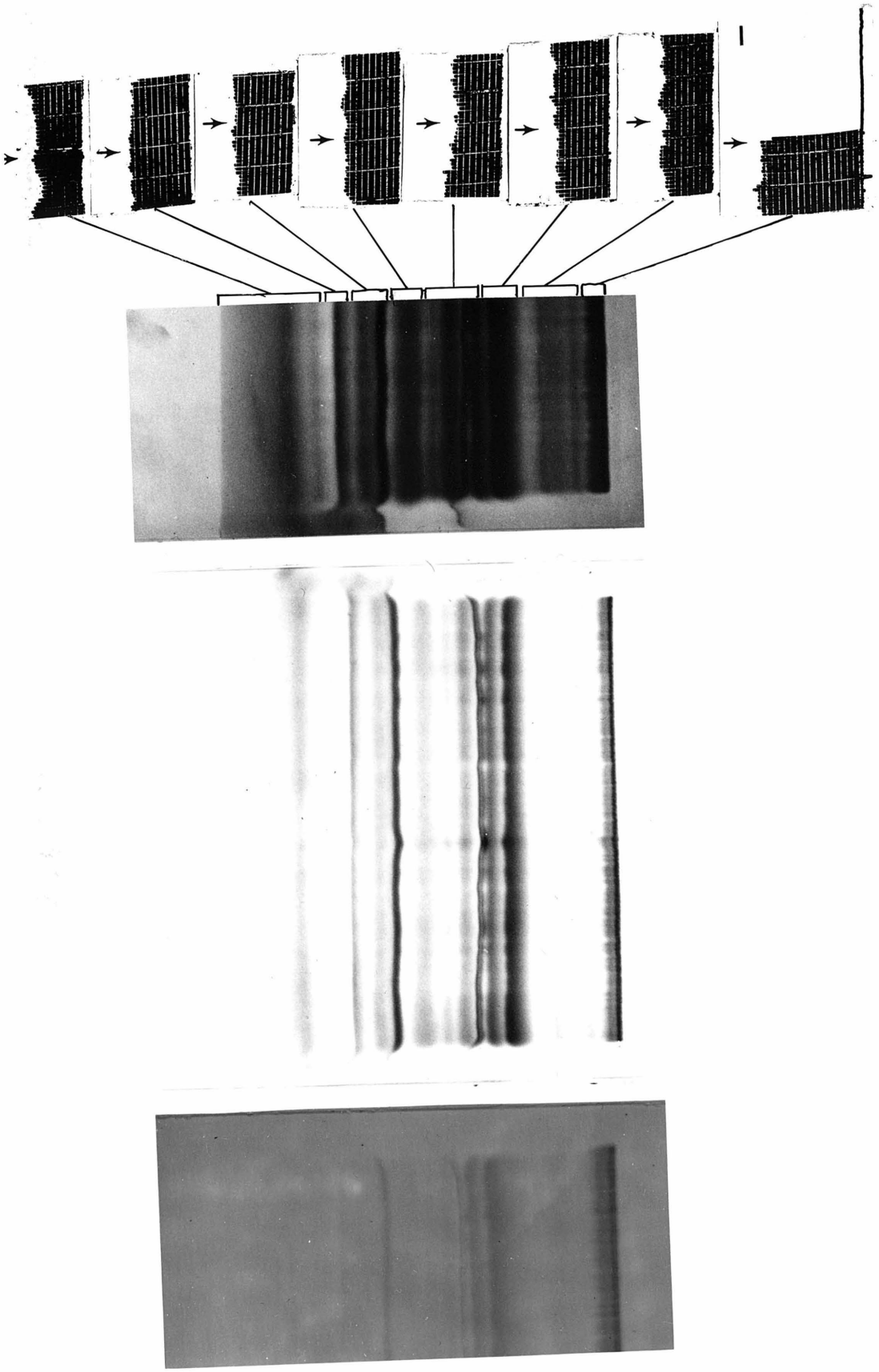


Figure 10

Figure 11. Silica gel TLC-purified conusine on Sephadex G-10.

Conusine in this experiment was obtained from 18 Conus (average length, 27 mm) and had been fractionated on Sephadex G-50 in 20 mM ammonium acetate, pH 8.3, and further purified by TLC on 0.5 mm silica gel plates in ethanol-ammonium hydroxide-water, 7:2:1. This conusine sample had been used for NMR, UV, and IR studies and was recovered from the last of these in KBr solution. To desalt the conusine, the sample was loaded on a calibrated Sephadex G-10 column, equilibrated with 20 mM ammonium acetate, pH 8.3, and was eluted with the same buffer. Fractions (2.1 ml each) were lyophilized individually and redissolved in 100 μl ASW each for bioassay on clam heart. The activity profile is a composite, representing the average of results obtained from several hearts. The traces shown were obtained from a single heart at a dilution of 1:100. Calibration: 30 sec, 0.1 g.

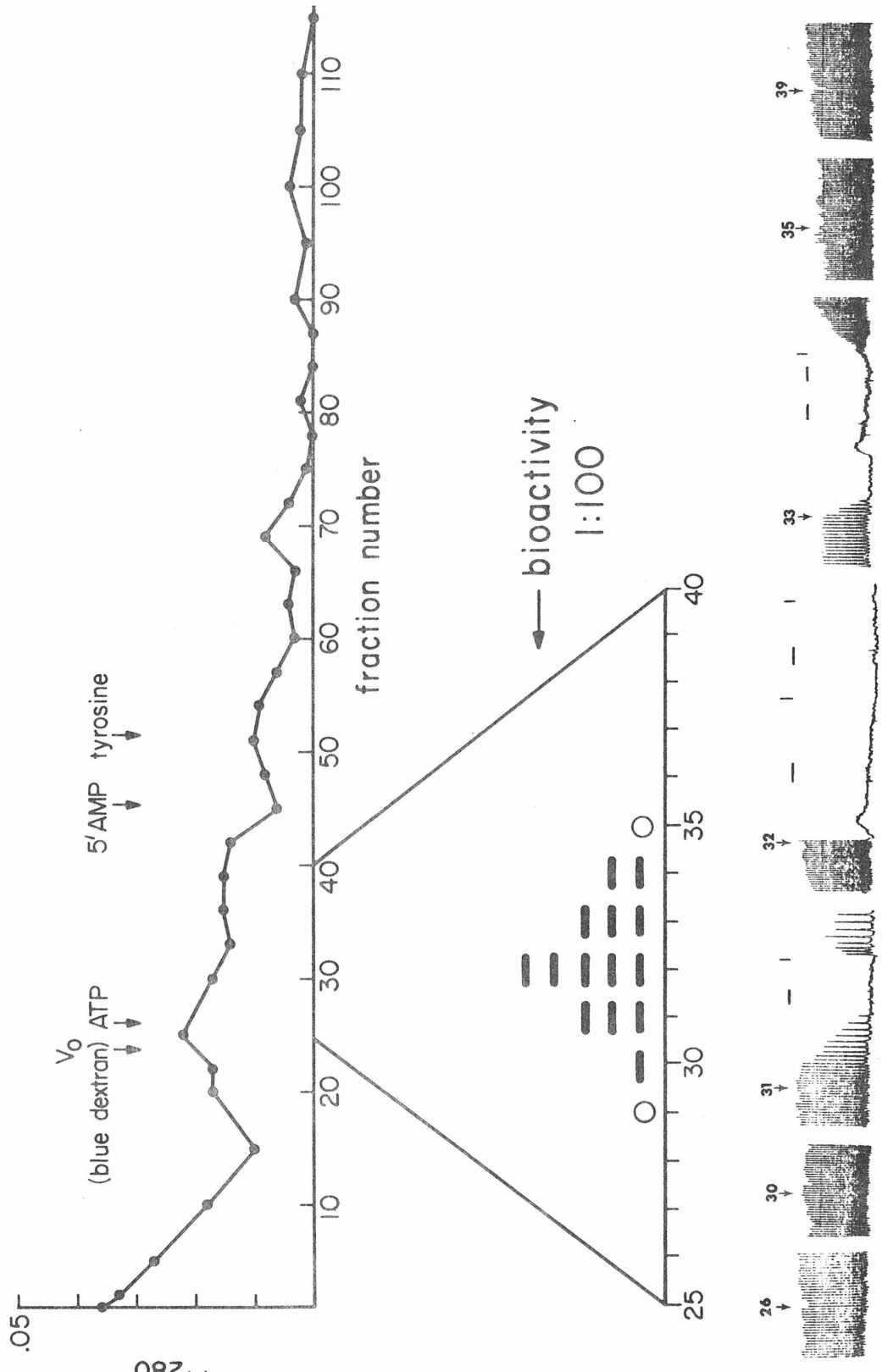

A280
.05
Vo
(blue dextran)
ATP
5'AMP
tyrosine
fraction number
bioactivity
1:100
10
20
30
40
50
60
70
80
90
100
110
25
26
30
31
32
33
35
39

Figure II

Figure 12. Analytical cellulose TLC of G-50-purified conusine and silica gel TLC-purified conusine.

Three 0.16 mm cellulose-coated strips were loaded with (1) G-50-purified conusine (G-50 III), (2) silica gel TLC-purified conusine (sg TLC 1) and (3) an ammonium acetate extract of a blank silica gel TLC plate, containing the organic binder used in the preparation of the plates. Each of the cellulose strips was then developed in one of the three solvent systems, A, B, or C, described in Figure 26. The strips were air dried and stained in an iodine vapor tank.

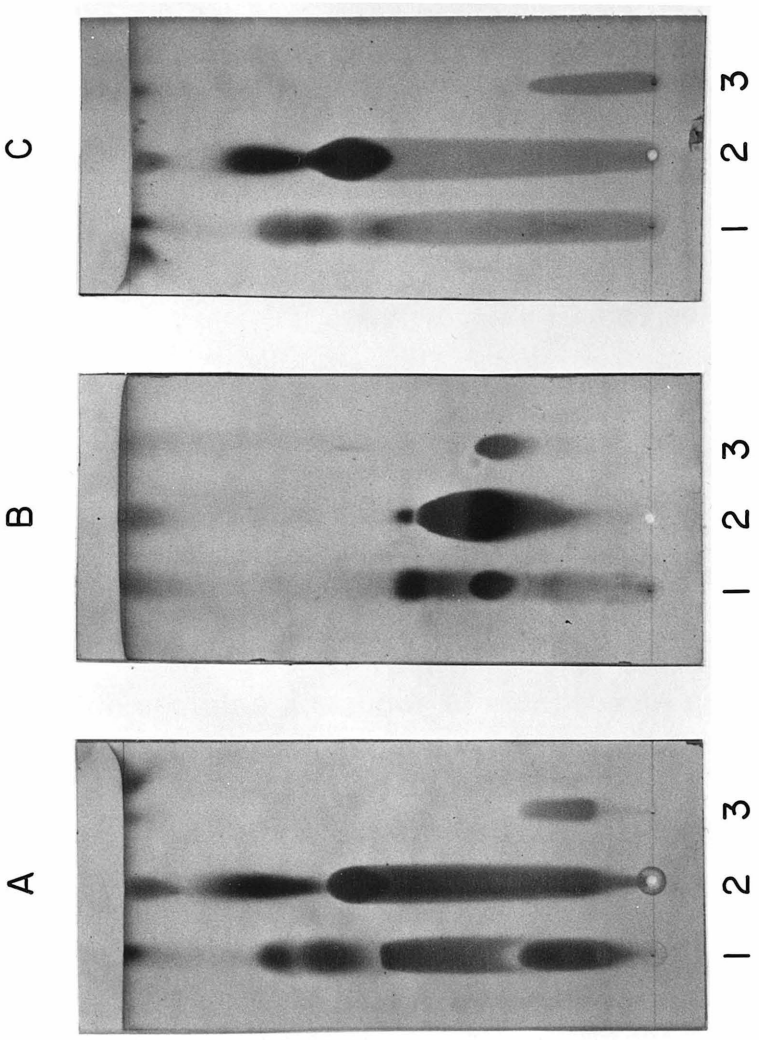


Figure 12

Figure 13. Analytical TLE of ACh and conusine.

1-2 μl aliquots of (1) ACh, (2) G-50-purified conusine, and (3) silica gel TLC-purified conusine were loaded onto an Eastman cellulose chromagram sheet which had been equilibrated in pyridine-acetate buffer, pH 6.5 and pre-electrophoresed 10 min at 390 V. The sheet was then electrophoresed at 390 V. for 60 min for pH 6.5 pyridine-acetate as the reservoir buffer, and air-dried and stained in an iodine vapor tank. The photograph shows that G-50-purified conusine, which contains a number of components not present in silica gel TLC-purified conusine (see Figures 26, 29), is not resolved into these components by TLE, and runs approximately with the silica gel TLC-purified conusine, with a slight bit more tailing. Both conusine samples travel toward the cathode and show a greater electrophoretic mobility than CAh.

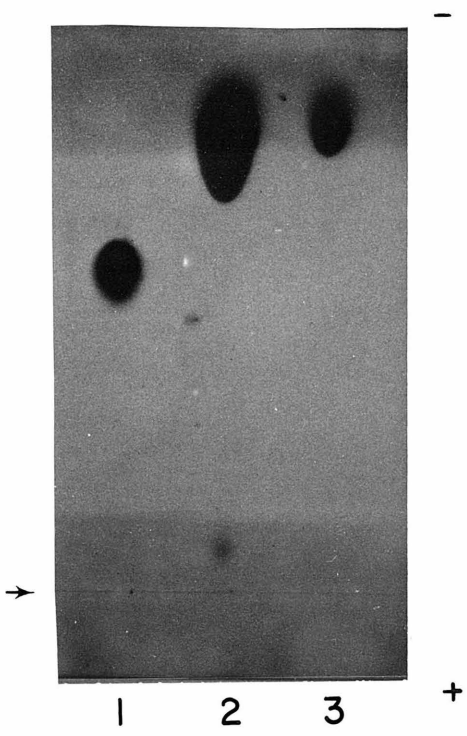


Figure 13

Figure 14. Semi-preparative cellulose TLC of silica gel-purified conusine.

In the experiment illustrated in this figure, the venom from 104 Conus, 22.2 mm average length, was purified by G-50 fractionation and semi-preparative silica gel TLC. The active band (sg TLC 1) from the silica gel TLC was extracted with water, lyophilized (giving about 6 mg of material), dissolved in methanol and loaded on a 0.5 mm cellulose TLC plate. The plate was developed in ethanol-water, 7:3, for $2\frac{1}{2}$ hrs, air-dried and stained in an iodine vapor tank. The photo shows only one clearly detectable band, with an R_f of 0.72, other than the silica gel organic binder which remains at the origin. This band, as well as other regions of the TLC (including the solvent front which contained a number of solvent impurities and the region around the origin which contained the organic binder) were extracted from the cellulose with water, lyophilized, redissolved in 800 μl ASW, and bioassayed on a number of clam hearts at various dilutions. The traces show the assay on a single clam heart of 1:30 dilutions of extracts from the five TLC regions into which the plate was divided. The band with $R_f = 0.72$ can be seen to contain the conusine bioactivity. Calibration: 30 sec, 0.1 g.

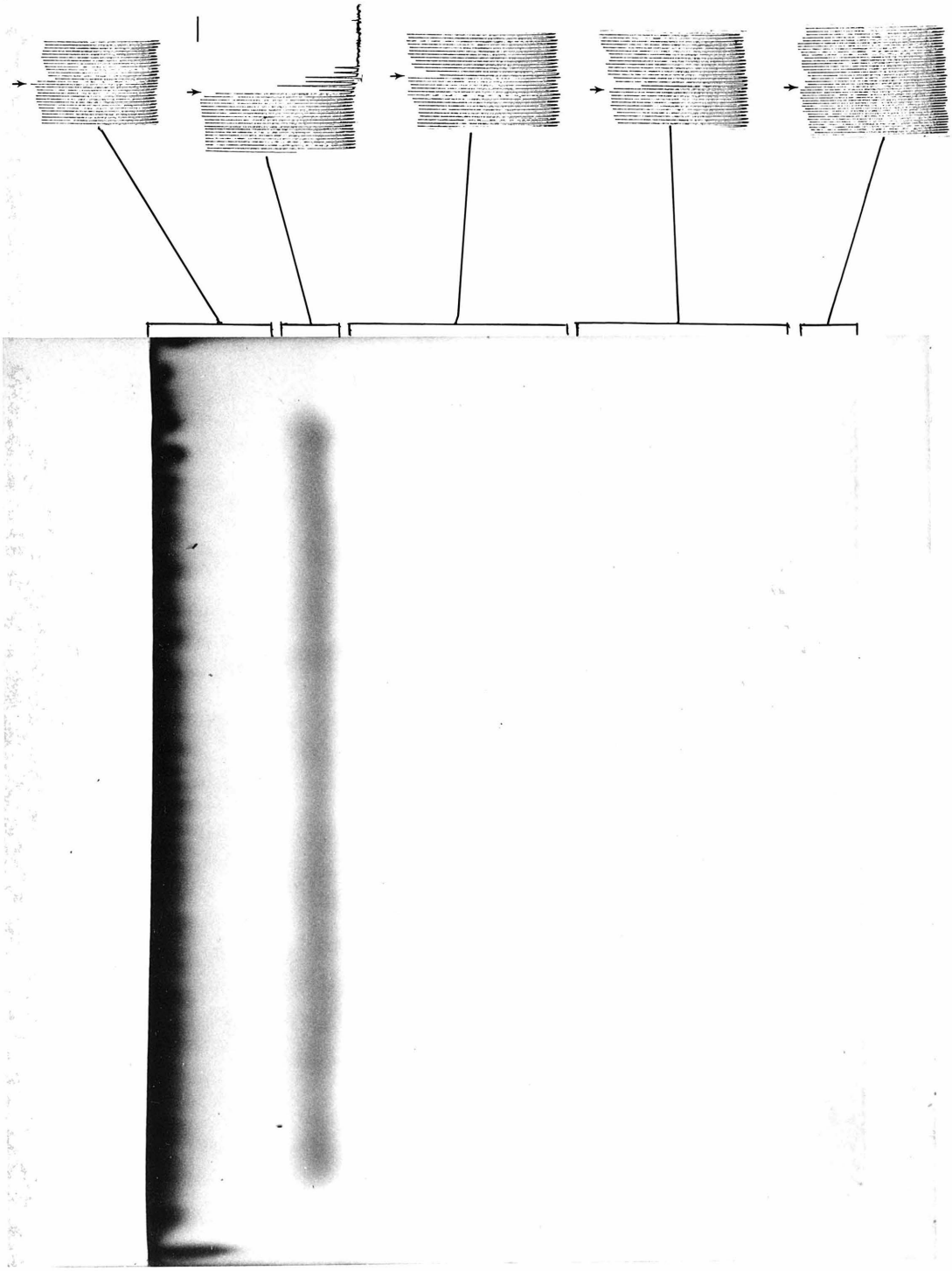


Figure 14

Figure 15. Analytical cellulose TLC of cellulose TLC-purified conusine.

The analytical cellulose TLC's of (1) silica gel TLC-purified conusine (sg TLC 1), (2) cellulose TLC-purified conusine (cell TLC 4), and (3) an aqueous extract of a blank cellulose plate, are shown. The three solvent systems A, B, and C are described in Figure 9. (1) and (2) were developed on the same cellulose TLC, while (3) was run on a separate chromatogram under the same conditions. All TLC's were air dried and stained in an iodine vapor tank. Comparison of (1) with (2) shows that the cellulose semi-preparative TLC purification step has removed one or possibly two components from sg TLC 1, while comparison of (2) with (3) indicates that some impurities were extracted from the semi-preparative cellulose plate and are present in cell TLC 4.

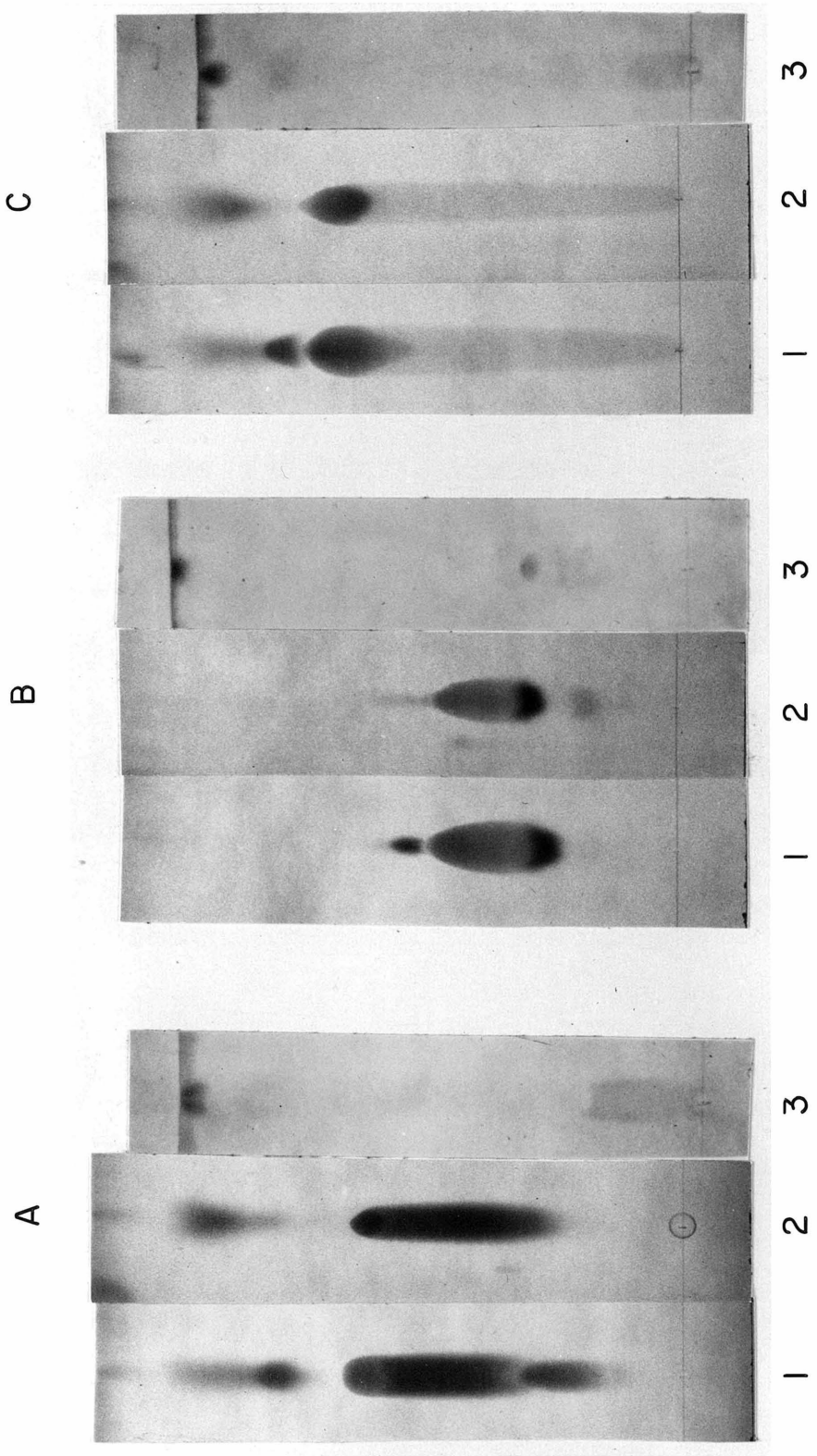


Figure 15

Figure 16. IR spectrum of conusine.

The IR spectrum of a KBr pellet of cellulose TLC-purified conusine is shown. The conusine sample was obtained from the cellulose TLC shown in Figure 14. The conusine-containing band was extracted with water, lyophilized, dissolved in d_6-D_2O and used to obtain the NMR spectrum shown in Figure 17, relyophilized, and finally dissolved in an aqueous solution containing 50 mg of KBr and lyophilized to give a homogeneous mixture of conusine sample + KBr. The reference beam in this spectrum was reduced by roughly 80% in order to magnify the very small absorptions present.

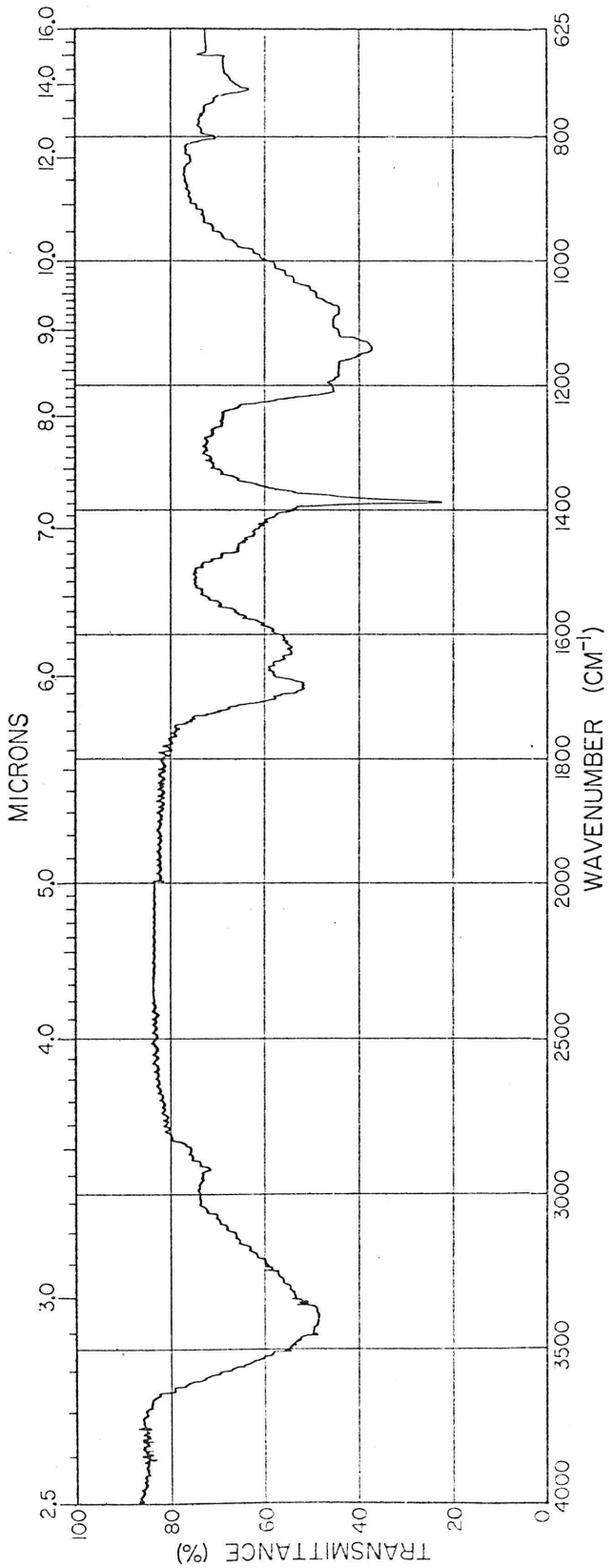

MICRONS
2.5
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
12.0
14.0
16.0
WAVENUMBER (CM⁻¹)
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
625
TRANSMITTANCE (%)
100
80
60
40
20
0

Figure 16

Figure 17. NMR ^{1}H spectrum of conusine.

The Fourier transform ^{1}H spectrum of cellulose TLC-purified conusine in D_2O is shown. Ethanol was added as a calibration standard to the sample (in a separate experiment; ethanol absorptions are not shown here) and the chemical shift values (τ) of the conusine absorption peaks were calculated using the methyl proton absorptions of the ethanol as a reference. The chemical shifts of the distinguishable conusine peaks, the approximate relative areas under the peaks, and some possible assignments are as follows:

peak	τ	relative area	possible assignments
1	5.93 5.99	4	$-C\underline{H}_2-O-\overset{O}{\overset{\|}{C}}-R$
2	6.67 6.72 6.79	4	$-C\underline{H}_2-OH$ $-C\underline{H}_2-\overset{+}{N}R_3$
3	7.05	16	$C\underline{H}_3-\overset{+}{N}R_3$
4	7.34	1	$-C\underline{H}_2-SH$ $-C\underline{H}_2-S-S-R$
5	8.41 8.49	2	$-C\underline{H}_2-C-OH$ $-C\underline{H}_2-C-NR_2$ $-C\underline{H}_2-C-\overset{+}{N}R_3$ $-C\underline{H}_2-C-O-\overset{O}{\overset{\|}{C}}-R$ $-C\underline{H}_2-C-SH$

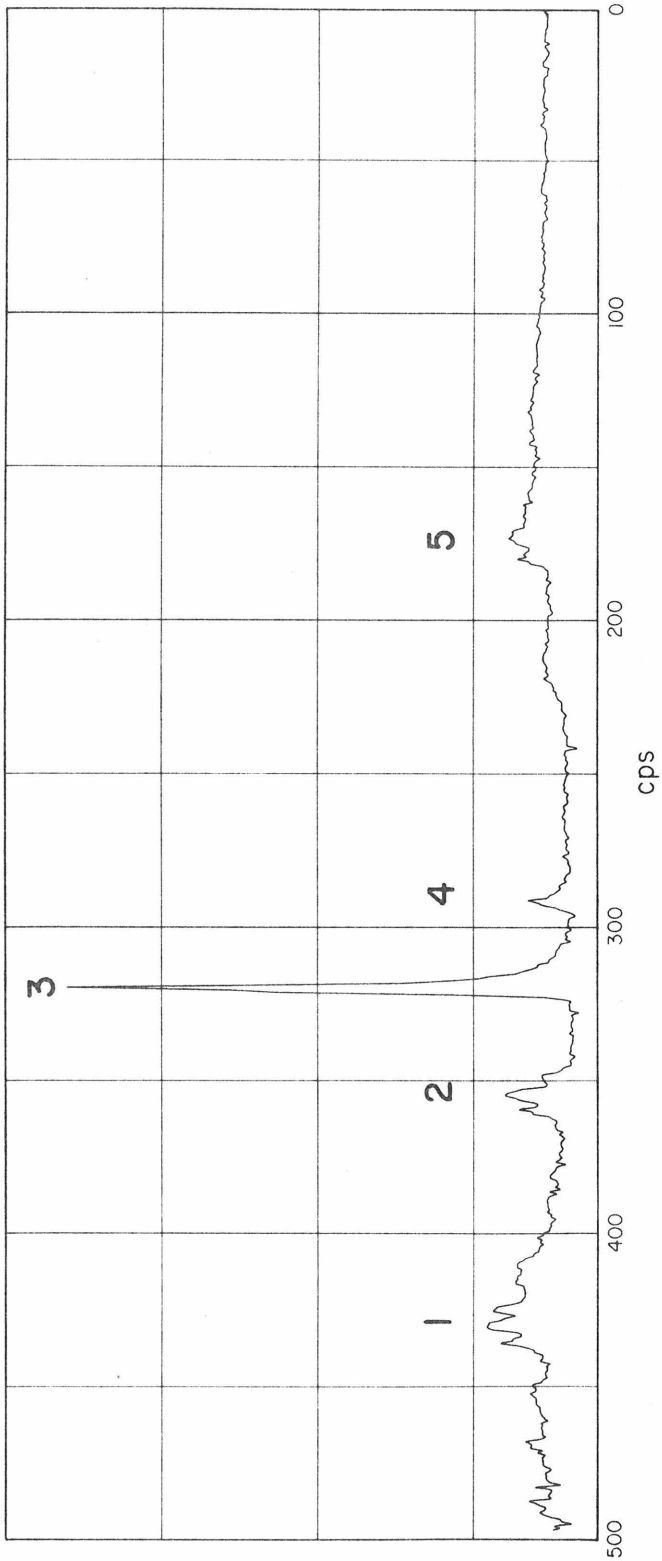


Figure 17

Figure 18. Inhibition of the spontaneously beating *in vitro* clam heart by conusine and ACh.

The inhibitory effect of a series of concentrations of (a) conusine and (b) ACh is shown on two different hearts. The conusine sample used in (a) is a mixture of G-50-purified conusine from two extractions (one of 53 *Conus*, 25 mm average length, 21 mg lyophilized weight of G-50 III, and the other of 65 *Conus*, 16 mm average length, 11.5 mg lyophilized weight of G-50 III). The concentration of the sample is 57 venom ducts/ml, or 16.2 mg/ml of material. Thus, in this preparation, a concentration of about 6×10^{-3} ducts/ml (in a bath of 1.5 ml, approximately 1% of the total conusine from one snail) is sufficient to inhibit totally the clam heart. In terms of dry weight of G-50-purified conusine, 1.6 μg/ml is sufficient to inhibit totally the heart. If a molecular weight of 400 for conusine is assumed, and if all the material present in the G-50 III sample is assumed to be conusine, a concentration of 4×10^{-6} M is obtained as an upper limit approximation of the molar concentration of conusine sufficient to inhibit totally the clam heart. The effective concentrations of both conusine and ACh vary from heart to heart within a range of a factor of about 10. The two hearts shown are representative of the average sensitivity to both conusine and ACh. Horizontal dashes on the traces represent ASW rinses of 15 ml. The small vertical lines indicate the addition of 5-HT, sufficient to give a final concentration of 10^{-7} g/ml or $\sim 2.6 \times 10^{-7}$ M. Calibration: 1 min, 0.1 g (force of contraction).

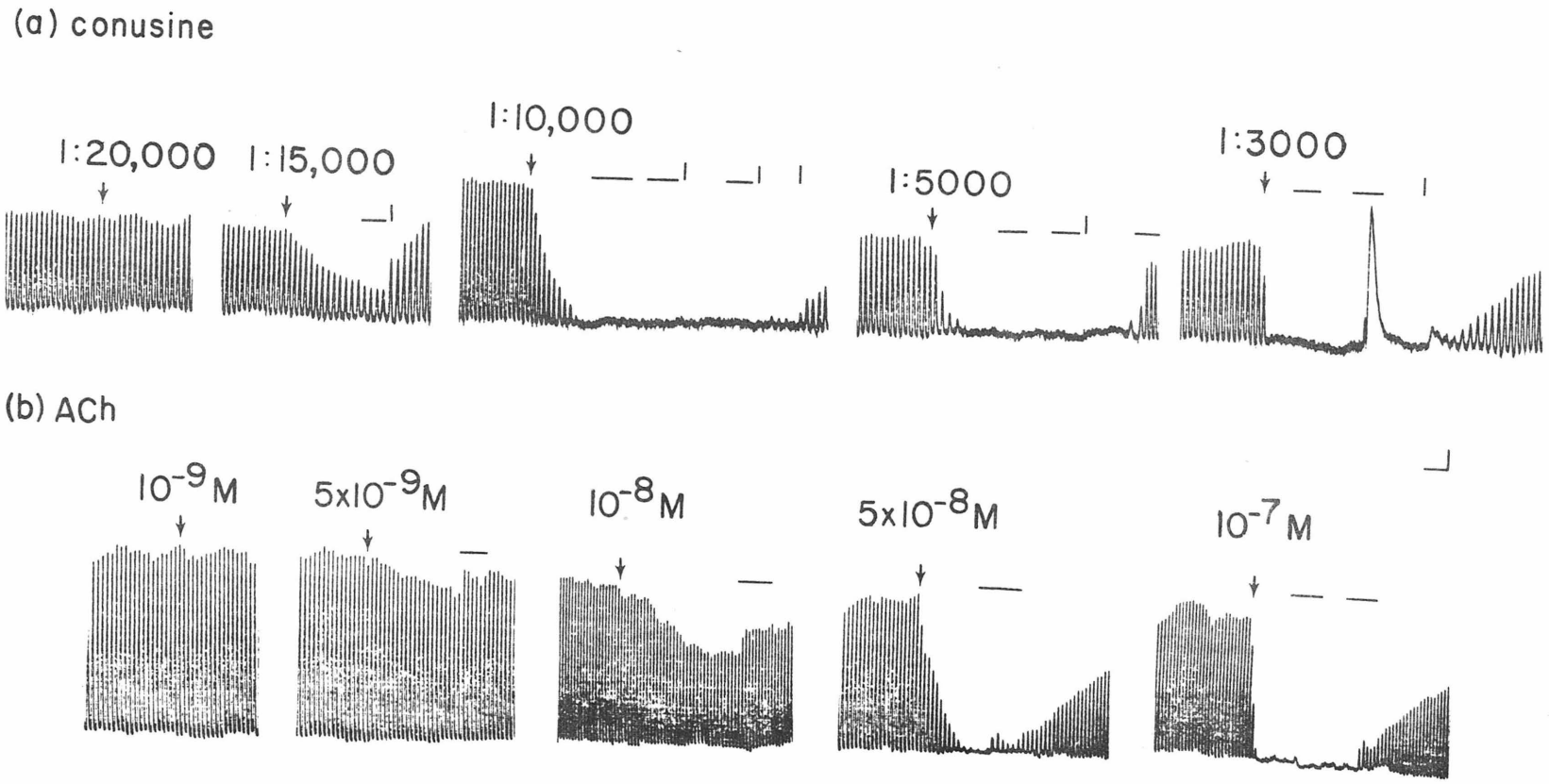

(a) conusine
1:20,000
1:15,000
1:10,000
1:5000
1:3000
(b) ACh
10^{-9} M
$5x10^{-9}$M
10^{-8}M
$5x10^{-8}$M
10^{-7} M

Figure 18

Figure 19. Antagonism of the action of conusine and ACh on clam heart by TEA.

The antagonism by a series of increasing concentrations of TEA of the action of conusine and ACh on two different clam hearts is shown. Recovery from TEA blockade of both conusine and ACh action was very slow in all hearts tested. In the trace shown here, the conusine response is still blocked to a great degree after eight 15 ml rinses over a period of 37 min, following application of 10^{-4} M TEA. Calibration: 1 min, 0.1 g.

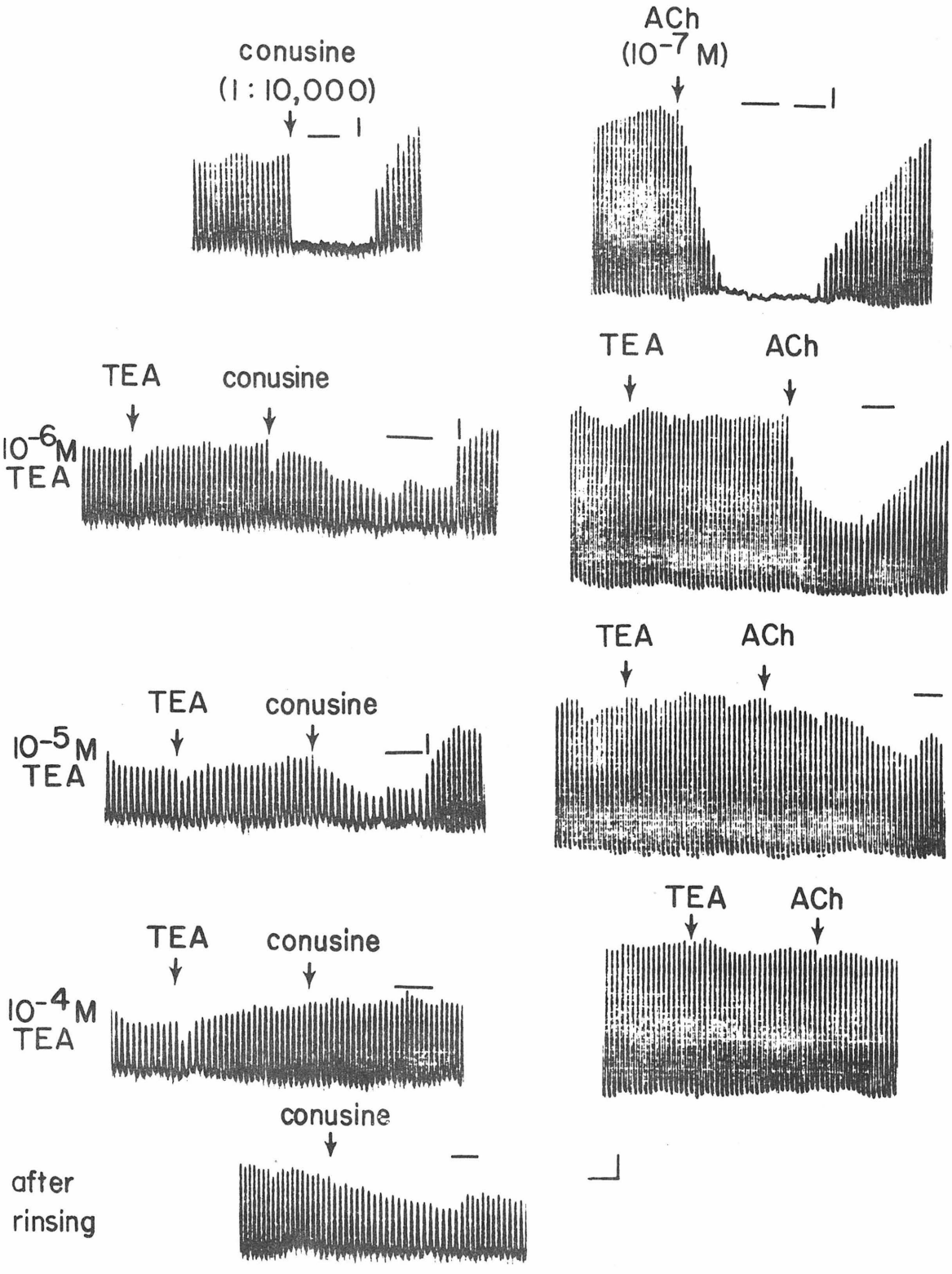

conusine
(1 : 10,000)
ACh
(10^{-7} M)
TEA
conusine
TEA
ACh
10^{-6}M
TEA
TEA
conusine
TEA
ACh
10^{-5}M
TEA
TEA
conusine
TEA
ACh
10^{-4}M
TEA
conusine
after
rinsing

Figure 19

Figure 20. Antagonism of the action of conusine and ACh on clam heart by BZQ.

The antagonism by very low concentrations of BZQ of conusine and ACh action on two different hearts is shown. As was true for TEA, antagonism by BZQ was extremely slow to reverse. In the trace shown here, the conusine response has not returned to normal after seventeen 15 ml rinses over a period of 1 hr, 45 min, following application of 5×10^{-7} M BZQ. Calibration: 1 min, 0.1 g.

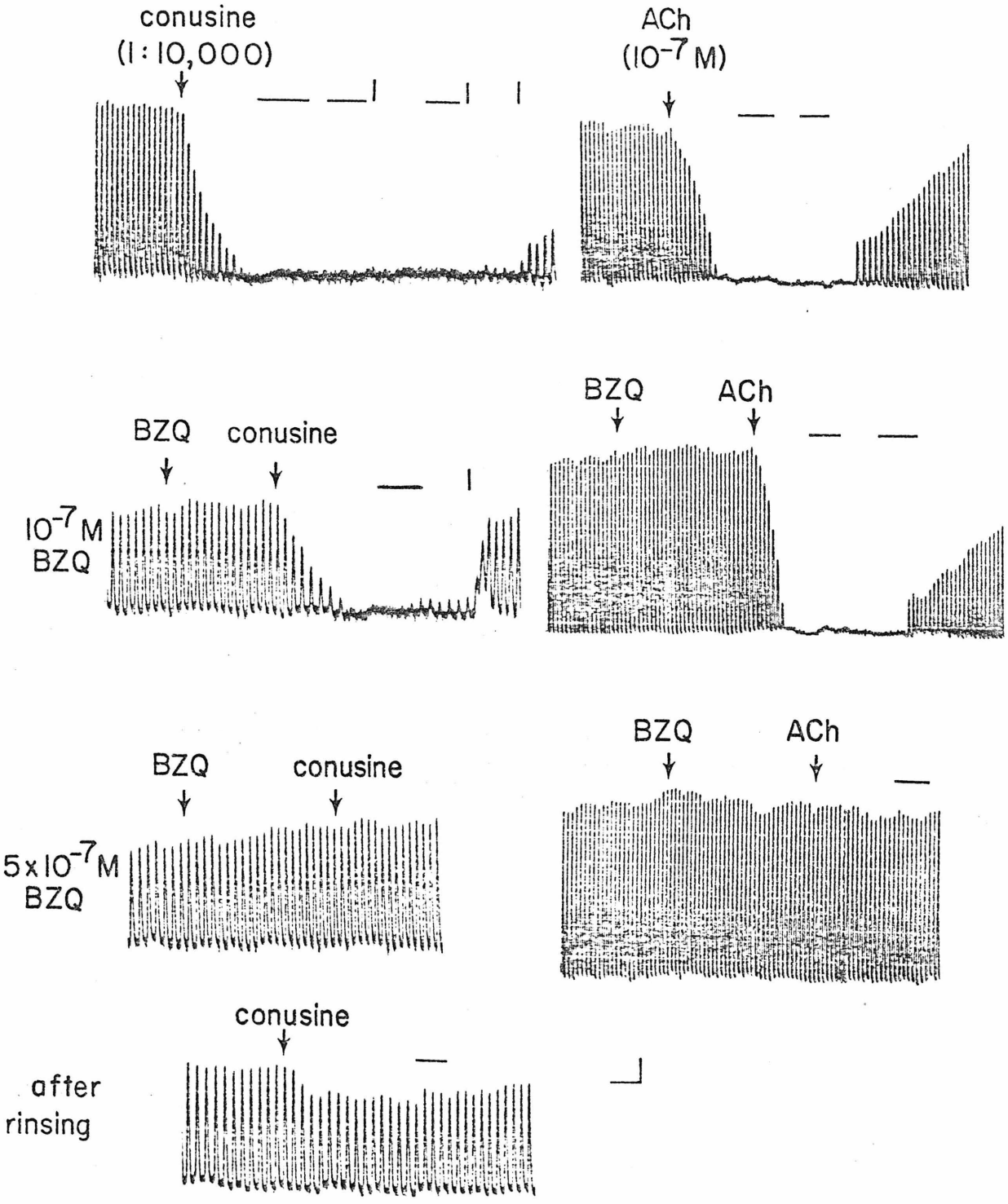

conusine
(1:10,000)
ACh
(10^{-7} M)
BZQ
conusine
10^{-7} M
BZQ
BZQ
ACh
BZQ
conusine
5×10^{-7} M
BZQ
BZQ
ACh
conusine
after
rinsing

Figure 20

Figure 21. Failure of dTC to block conusine or ACh action on clam heart.

The effect of a series of increasing concentrations of dTC on conusine and ACh action on two different hearts is shown. Even at the extremely high concentration of 10^{-3}M, dTC shows no antagonism of either the conusine or ACh response. Calibration: 1 min, 0.1 g.

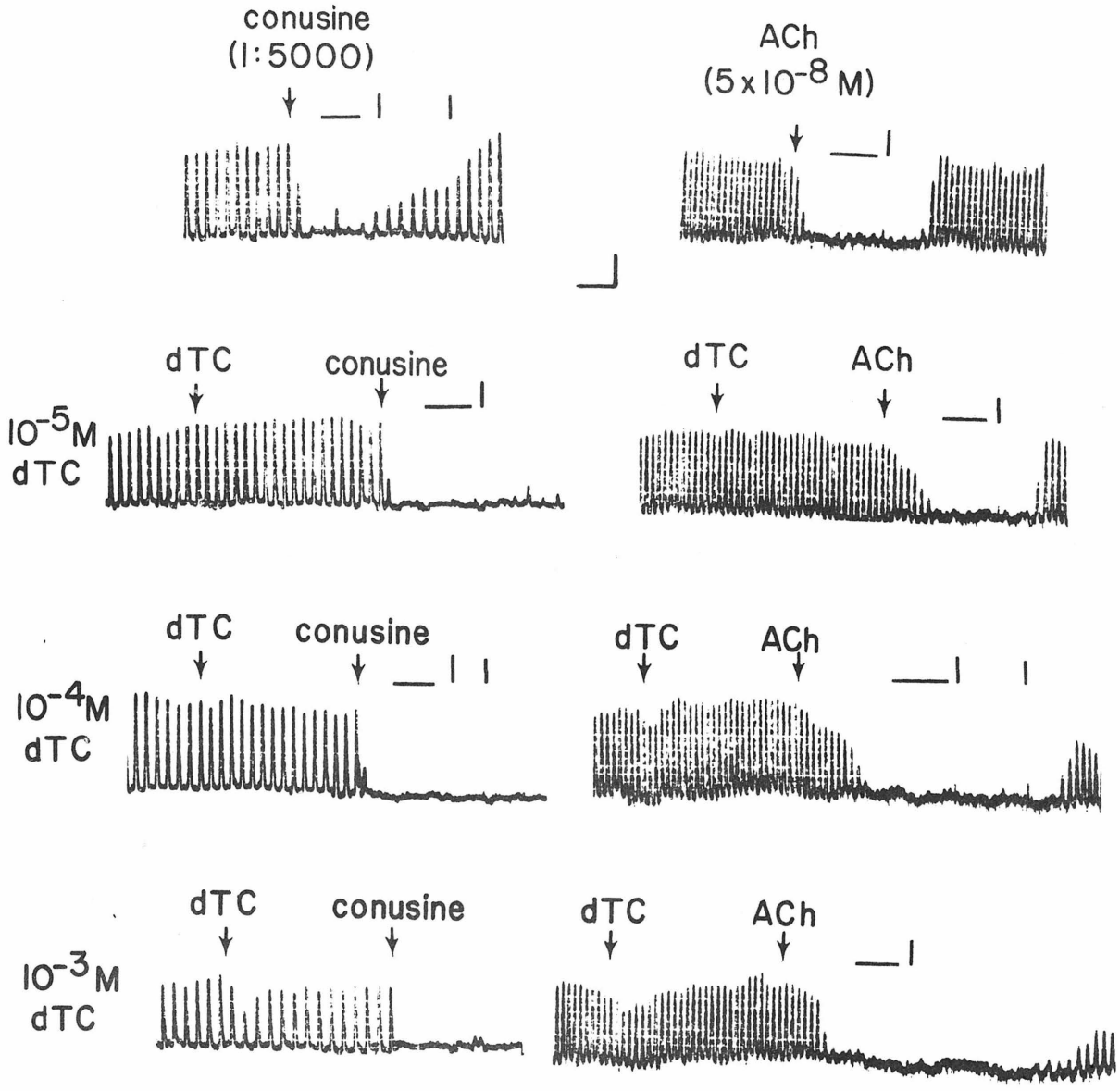
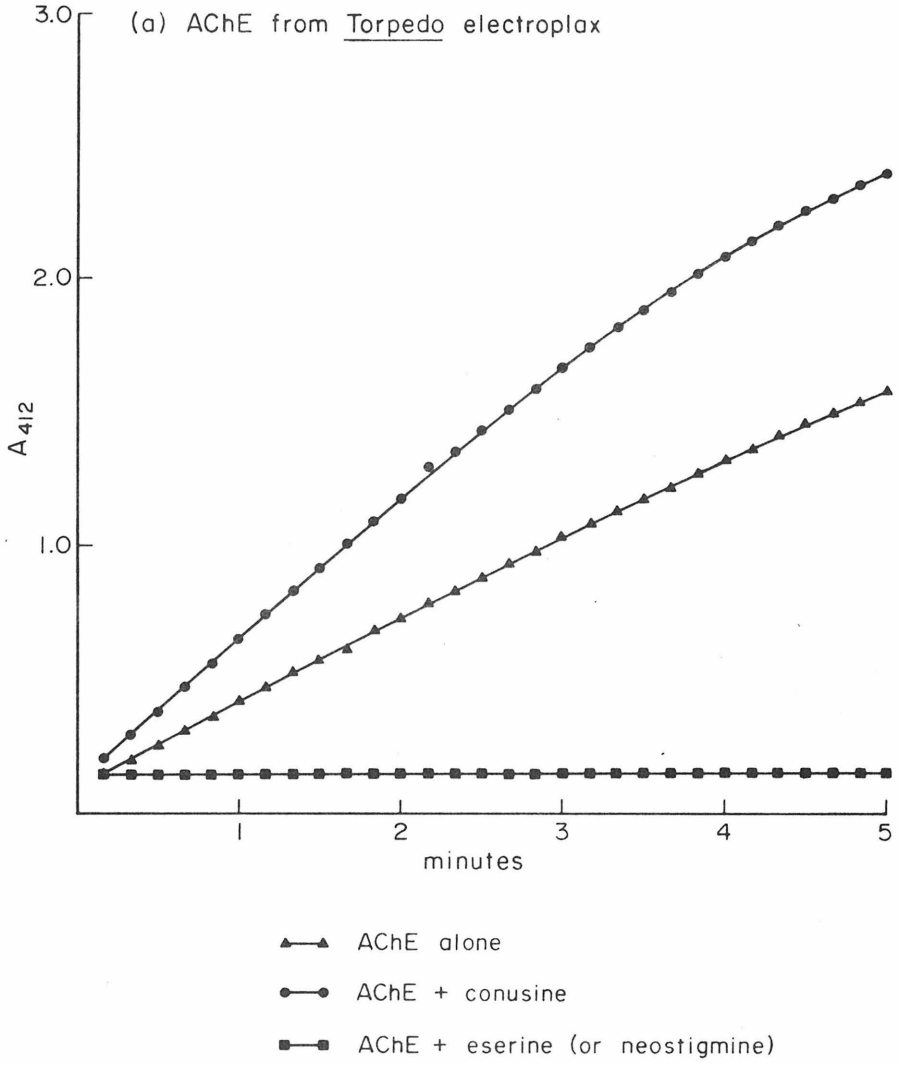

conusine
(1:5000)
ACh
(5 x 10-8 M)
dTC
conusine
dTC
ACh
10-5M
dTC
dTC
conusine
dTC
ACh
10-4M
dTC
dTC
conusine
dTC
ACh
10-3M
dTC

Figure 21

Figure 22. Effect of conusine on in vitro AChE activity.

AChE extracts from the electric ray Torpedo californica (a) and from Mercenaria heart (b) were assayed for esterase activity according to the spectrophotometric method of Ellman et al. (22, see Methods), in which the hydrolysis of acetylthiocholine is monitored by the change in absorption at 412 nm. Activity was assayed with and without the addition of 30 μl aliquots of conusine to the respective AChE extracts, to give a final concentration of conusine equal to that totally inhibitory to the spontaneously beating clam heart. The graphs show that conusine does not inhibit AChE activity from either source. In the case of Torpedo AChE, conusine appears to potentiate esterase activity. Conusine alone showed no cholinesterase activity in the spectrophotometric assay. 10^{-4} M eserine and 10^{-4} M neostigmine both completely inhibit all AChE activity, reducing the A_{412} to blank levels. Graphs represent the average of 3 assays, except in the case of the eserine and neostigmine experiments, which are the average of 2 assays.


3.0
(a) AChE from Torpedo electroplax
2.0
1.0
A412
1
2
3
4
5
minutes
AChE alone
AChE + conusine
AChE + eserine (or neostigmine)

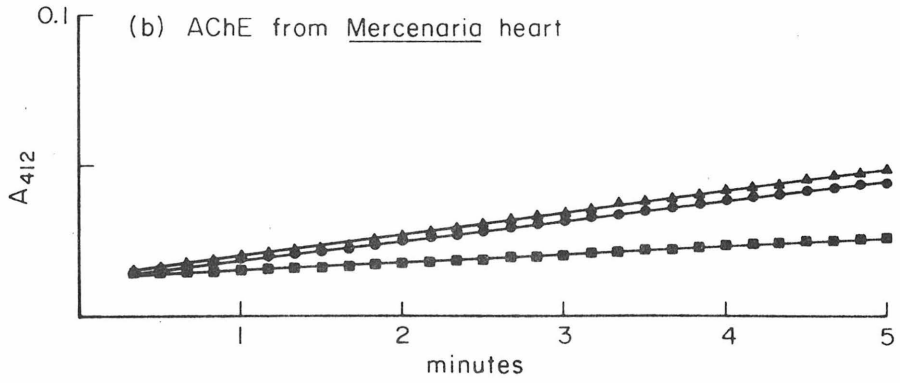

0.1
(b) AChE from Mercenaria heart
A412
1
2
3
4
5
minutes

Figure 22

Figure 23. Effect of eserine on ACh action on clam heart.

The effect of a series of increasing concentrations of eserine on ACh action is shown on two different hearts. Eserine does not exhibit a detectable potentiation of ACh action at any concentration shown, but rather causes a blockade of ACh action, even at low eserine concentrations. In all cases, the concentration of ACh is 10^{-8} M. Calibration: 1 min, 0.1 g.

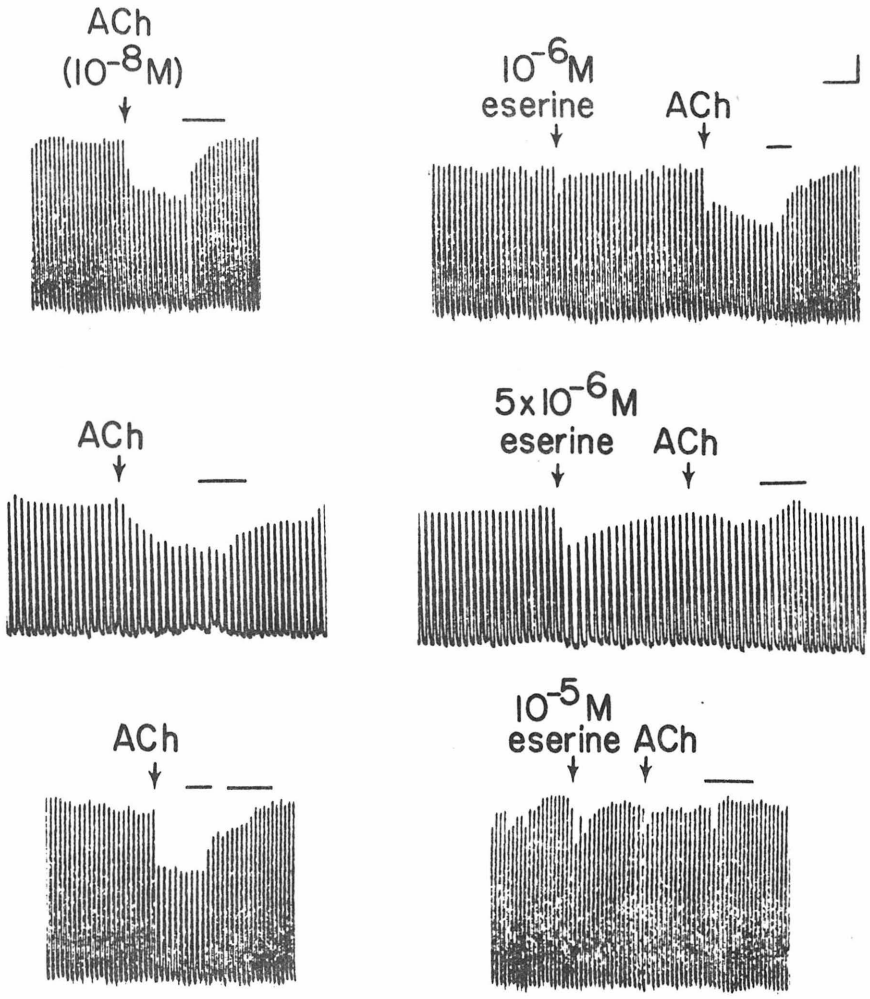

ACh
(10-8M)
10-6M
eserine
ACh
ACh
5x10-6M
eserine
ACh
ACh
10-5M
eserine
ACh

Figure 23

Figure 24. Iontophoretic application of (a) ACh and (b) conusine to medial cell of Aplysia pleural ganglion.

The responses of two different medial cells to (a) ACh and (b) conusine iontophoresed onto the soma are shown at three membrane potentials. ACh elicits a biphasic hyperpolarization, consisting of a rapid response followed by a slower one. At -50 mV the rapid phase of the response is barely distinguishable as a steeper initial slope in the hyperpolarization. At -83 mV, the rapid phase has inverted and is now depolarizing (an upward deflection in the trace) while the slow phase is absent entirely. At -105 mV, both phases have inverted and are depolarizing. Conusine elicits only the slow phase of the response, which is hyperpolarizing at -50 mV, depolarizing at -105 mV, and absent at its inversion potential, -83 mV. The small spikes in the ACh traces are the result of spontaneous presynaptic activation of the medial cell. Iontophoretic micropipettes were filled, respectively, with 1.0 M ACh and a concentration of silica gel TLC-purified conusine equal to 800 times that which was effective on clam heart. Calibration: 5 sec, 5 mV.

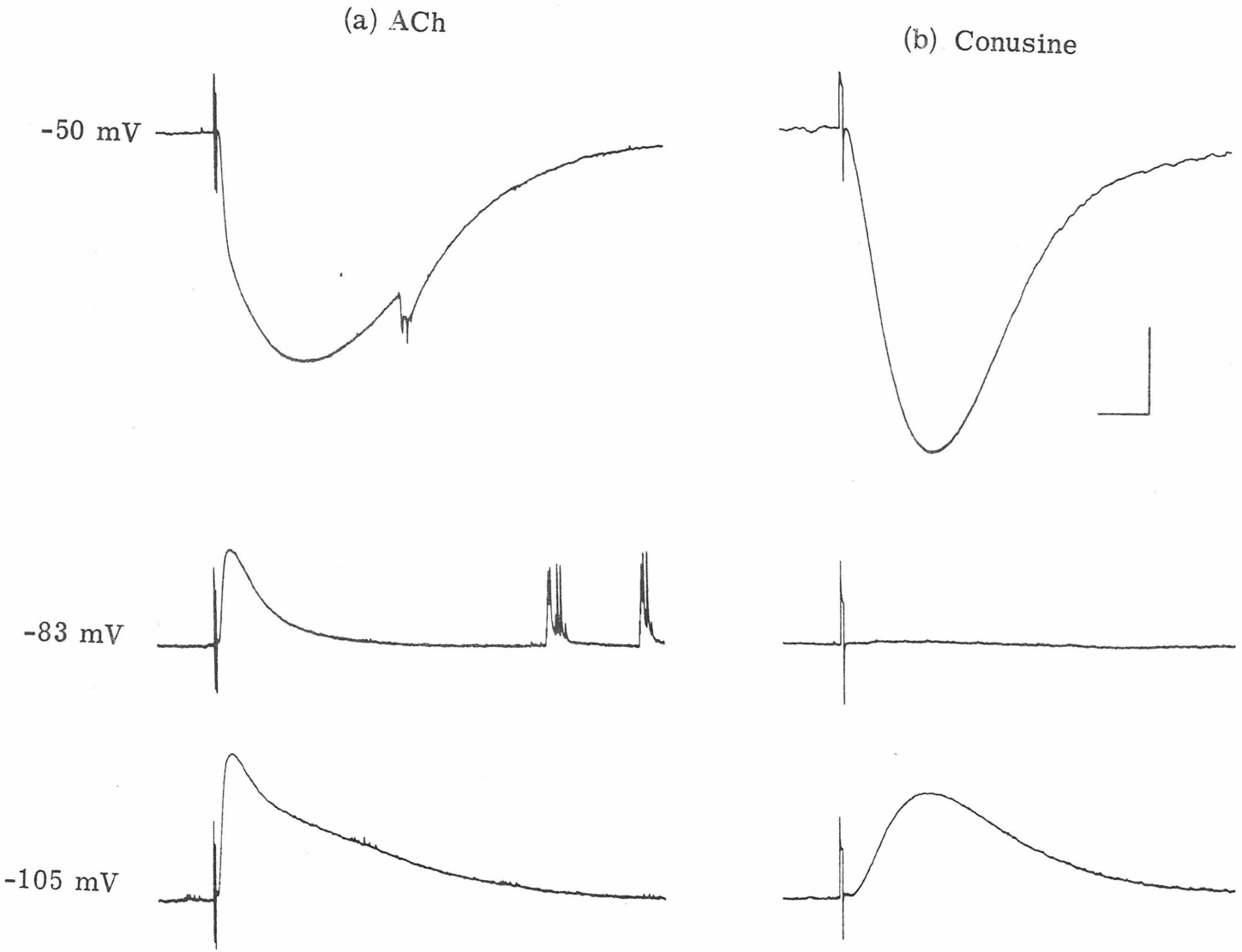


Figure 24

Figure 25. Antagonism by MXC of medial cell response to iontophoretically applied conusine.

The slow hyperpolarization of a medial pleural ganglion cell in response to iontophoretically applied conusine is shown at three membrane potentials, before and after addition of 10^{-3} g/ml MXC to the bath. MXC blocks completely the conusine response at all membrane potentials. Calibration: 5 sec, 5 mV.

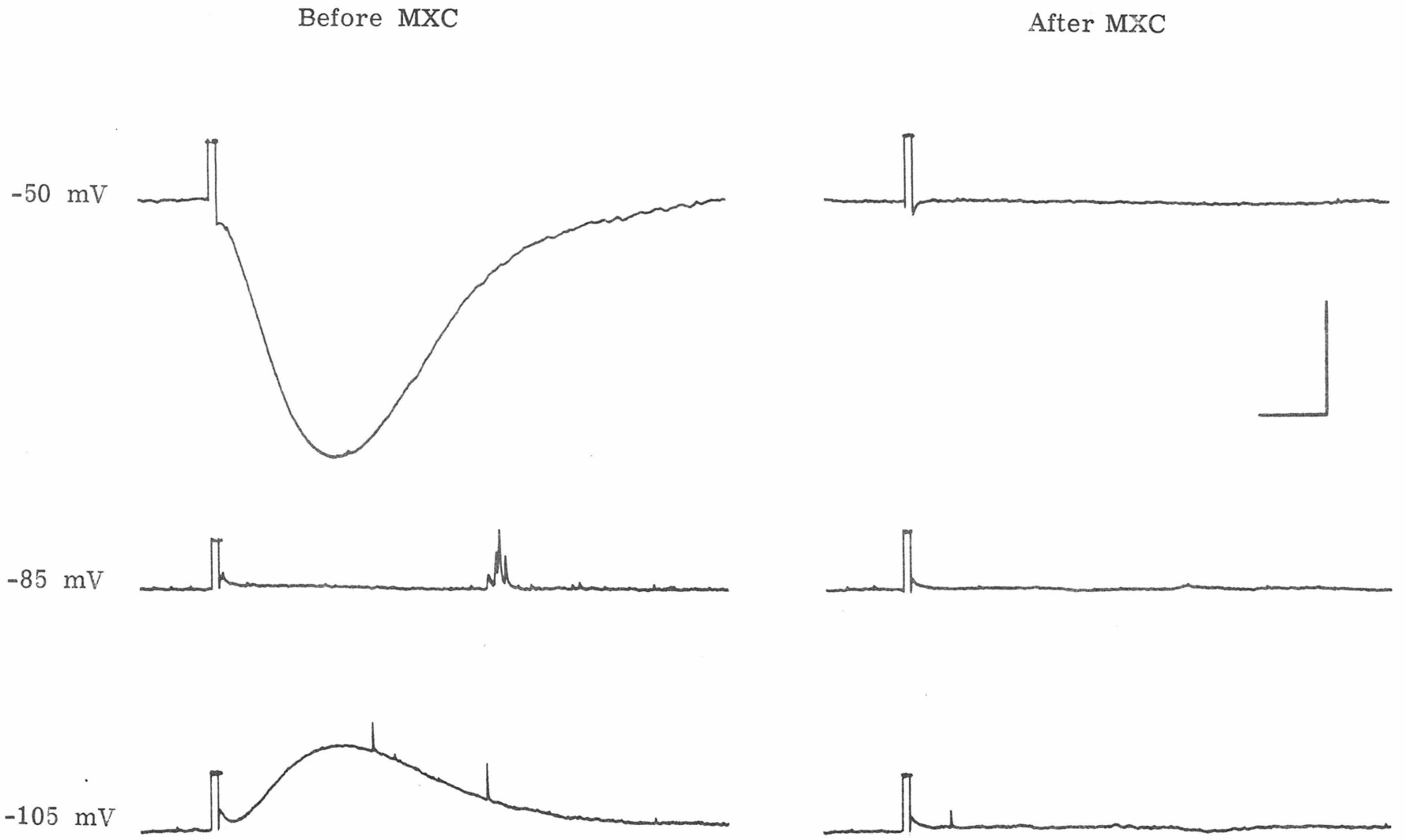


Figure 25

Figure 26. Antagonism by TEA of medial cell response to iontophoretically applied conusine.

The slow hyperpolarization of a medial pleural ganglion cell in response to iontophoretically applied conusine is shown at three membrane potentials, before and after addition of 10^{-3} g/ml TEA to the bath. TEA blocks completely the response to conusine at all membrane potentials. Calibration: 5 sec, 5 mV.

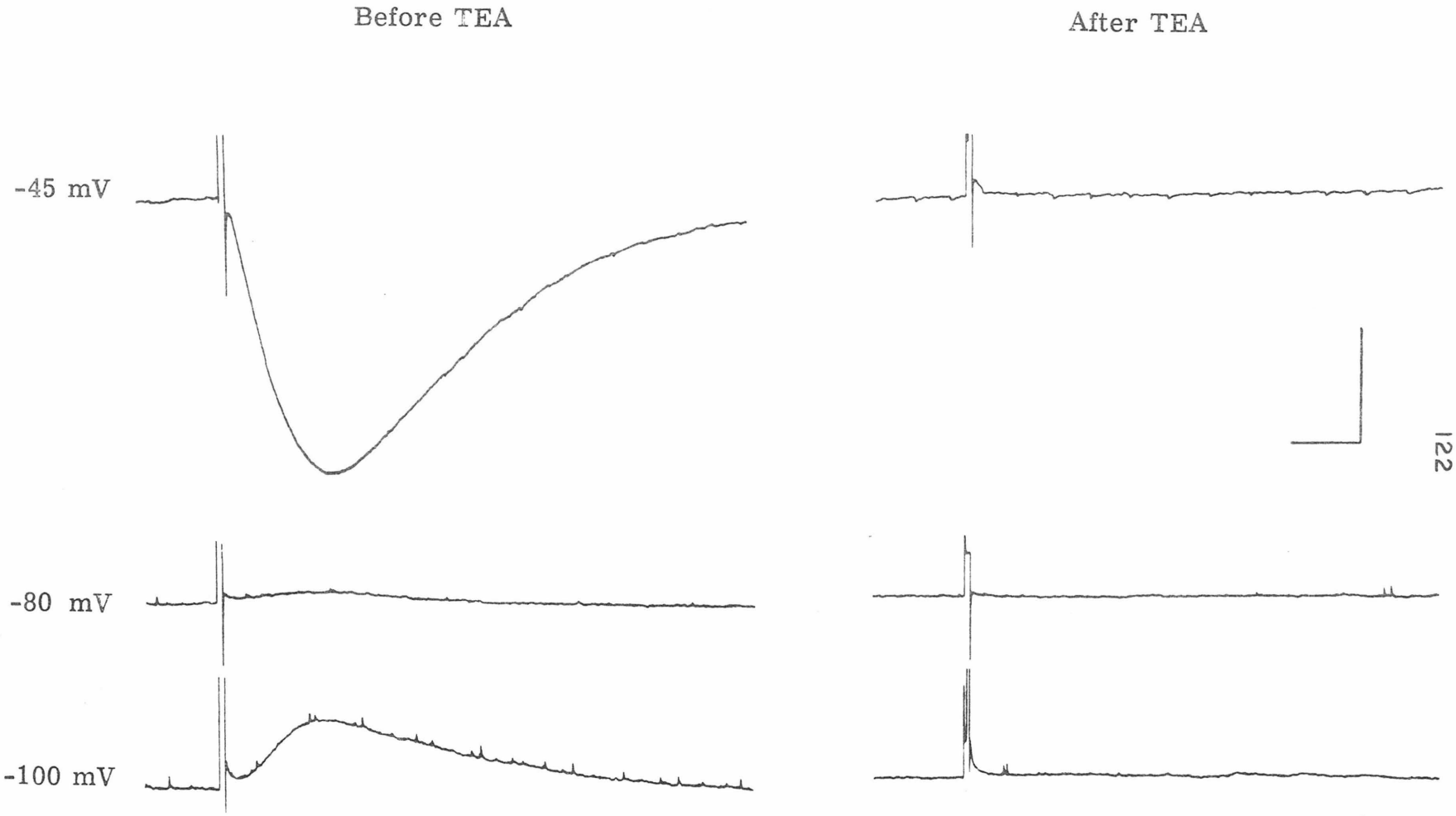


Figure 26

Figure 27. Effect of neostigmine on medial cell response to iontophoretically applied conusine.

The slow hyperpolarization of a medial pleural ganglion cell in response to iontophoretically applied conusine is shown at two membrane potentials, before and after the addition of 10^{-4} g/ml neostigmine to the bath. The second trace shows no significant effect of neostigmine immediately after its addition to the bath. The third trace shows partial antagonism of the conusine response by neostigmine within 10 to 15 min after its addition to the bath. Calibration: 5 sec, 5 mV.

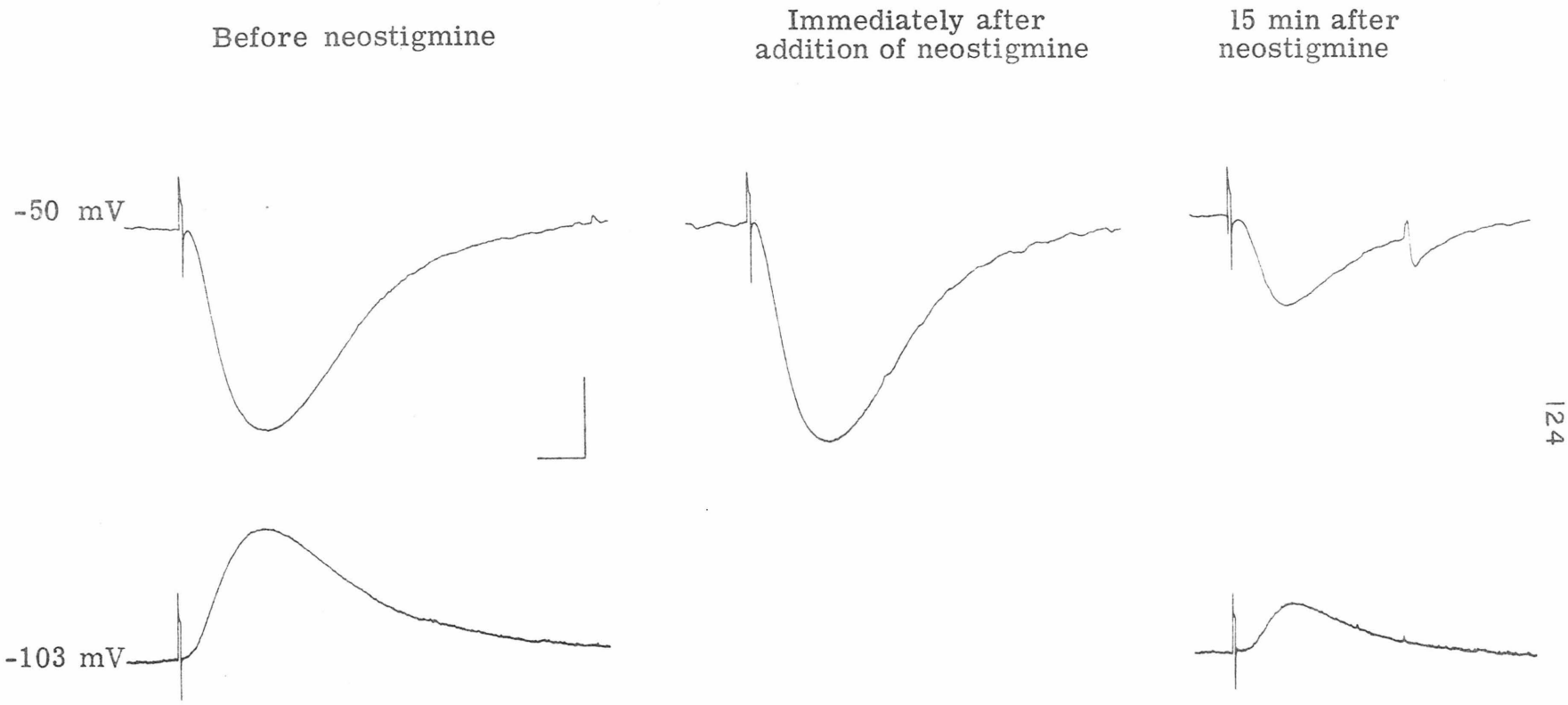

Before neostigmine
Immediately after addition of neostigmine
15 min after neostigmine
-50 mV
-103 mV

Figure 27

Figure 28. Effect of bath-applied conusine on medial, anterior and IPN 1 cells of Aplysia pleural ganglion.

A medial cell and the presynaptic cell IPN 1 were impaled and recorded from simultaneously as conusine (sufficient to give a final concentration approximately equal to that which was effective on clam heart) was added to the bath. Conusine caused an abrupt hyperpolarization to approximately -80 mV of the medial cell (bottom trace) and had no effect on the resting potential of IPN 1 (middle trace). In a separate experiment on a second ganglion, a medial cell and an anterior cell were both impaled and recorded from simultaneously as conusine (same concentration as above) was added to the bath. The conusine again caused hyperpolarization of the medial cell with no effect on the resting potential of the anterior cell (top trace). Calibration: 5 sec, 5 mV.

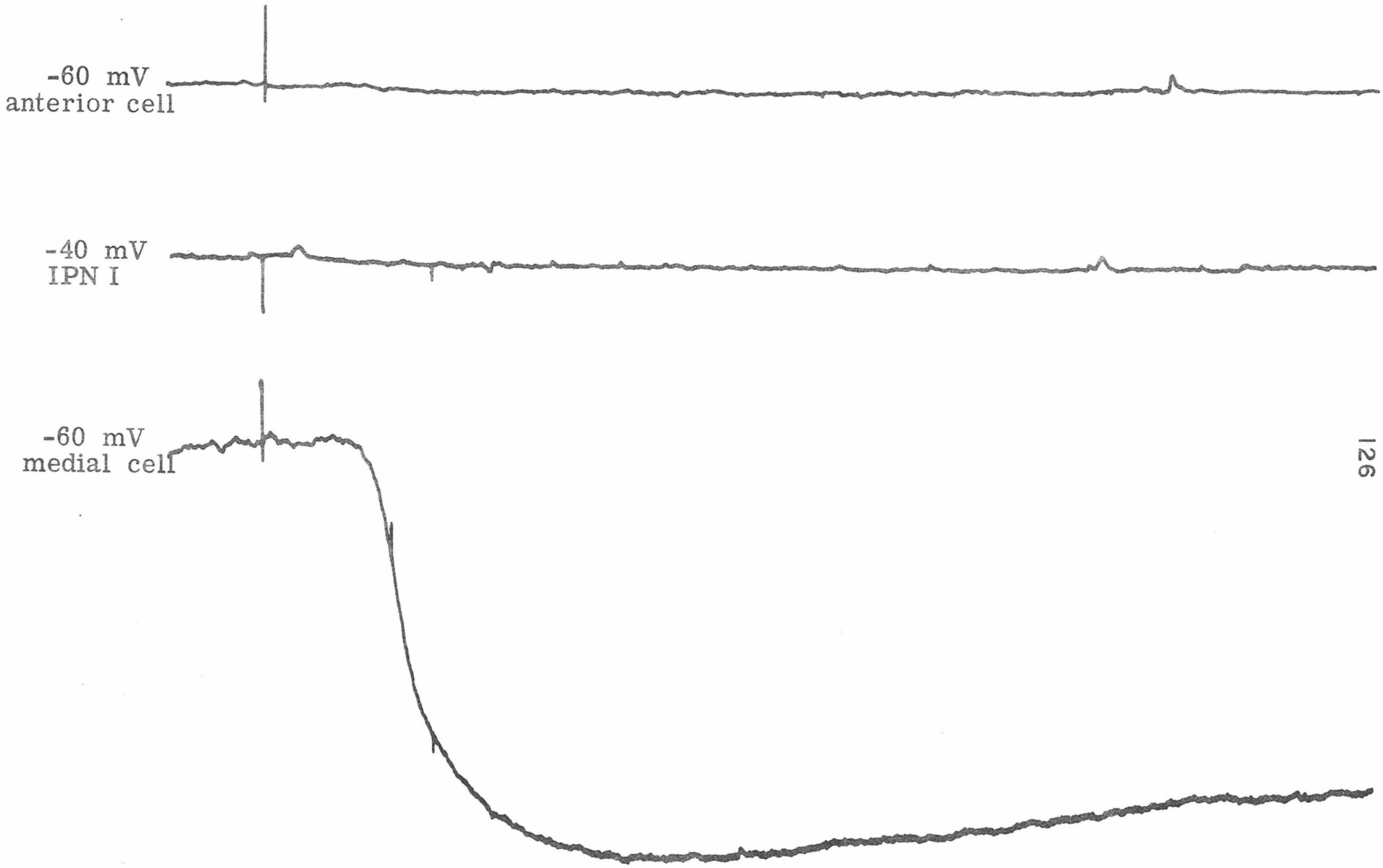


Figure 28

Figure 29. Effect of conusine on frog rectus abdominis and frog heart.

(a) Conusine, at a concentration 10 times that which completely abolishes the spontaneous beat of the clam heart, is shown to act as neither a cholinergic agonist nor antagonist on the frog rectus abdominis. The dashes on the traces indicate rinsing of the bath with 20 ml frog Ringer. Calibration: 1 min, 0.2 g.

(b) Conusine, again at a concentration 10 times that effective on clam heart, shows no effect on the cannulated, spontaneously beating frog heart. Inhibition of the frog heart by ACh is shown for comparison. The dashes on the traces indicate changing of the bath solution by perfusion with 30-50 ml frog Ringer from the reservoir. Calibration: 30 sec, 0.2 g.

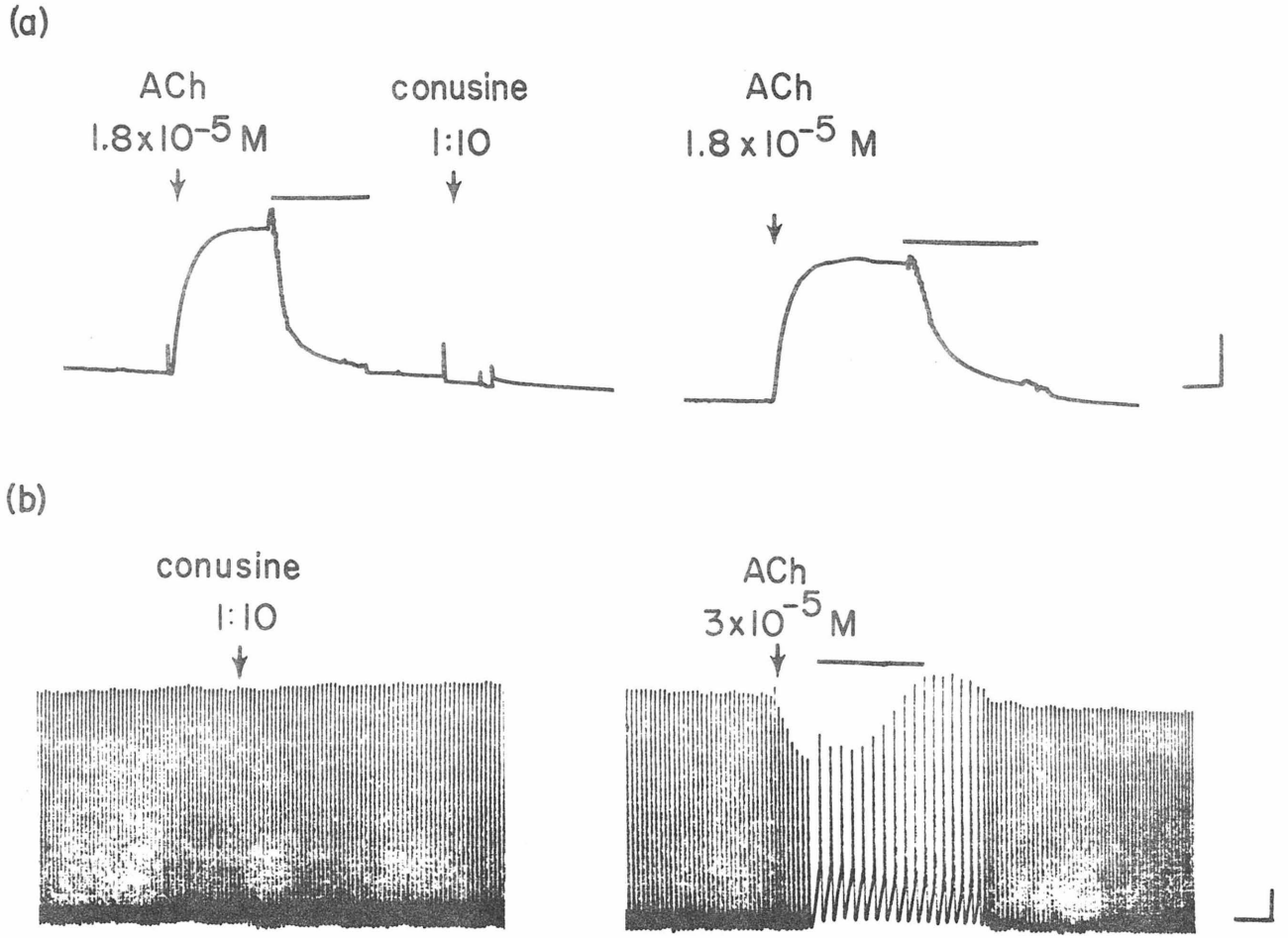

(a)
ACh
1.8x10-5 M
conusine
1:10
ACh
1.8 x 10-5 M
(b)
conusine
1:10
ACh
3x10-5 M

Figure 29

Figure 30. Effect of NTX on conusine and ACh action on clam heart.

The effects of increasing concentrations of NTX on the conusine response and of 10^{-3} M NTX on the ACh response of a single heart are shown. NTX at a very high concentration, 10^{-3} M, completely blocks conusine action on the heart but has no effect on the ACh response. The blockade by NTX of conusine action is easily washed out by a single 15 ml rinse. This result was confirmed in all four of the hearts tested. Calibration: 30 sec, 0.1 g.

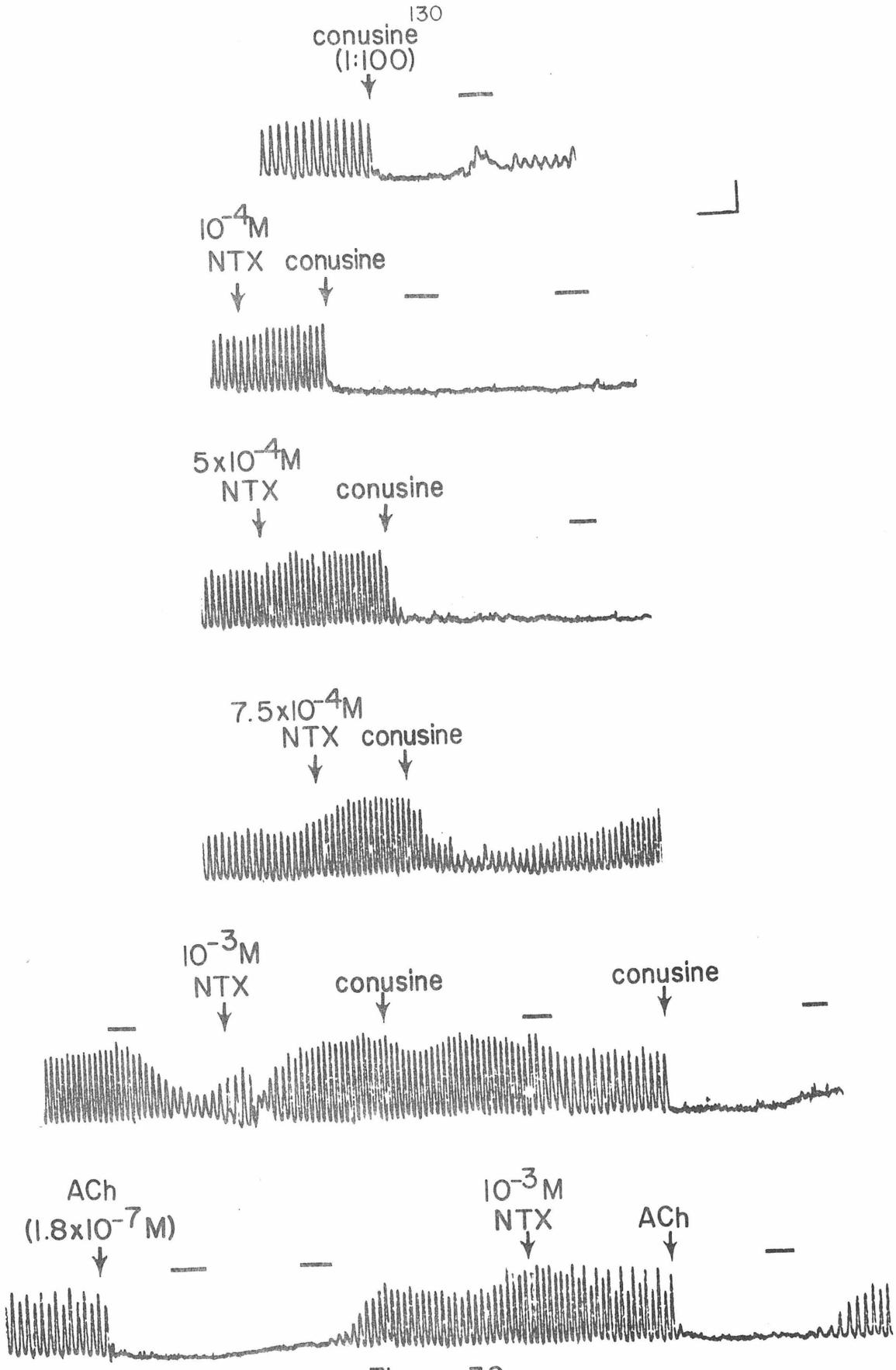

conusine
(1:100)
10^{-4}M
NTX
conusine
5×10^{-4}M
NTX
conusine
7.5×10^{-4}M
NTX
conusine
10^{-3}M
NTX
conusine
conusine
ACh
(1.8×10^{-7} M)
10^{-3}M
NTX
ACh

Figure 30

Figure 31. Effect of arecoline on clam heart.

The effect of a series of increasing concentrations of arecoline on a single clam heart is shown. Arecoline can be seen to be an effective cholinergic agonist at very low concentrations. Calibration: 1 min, 0.1 g.

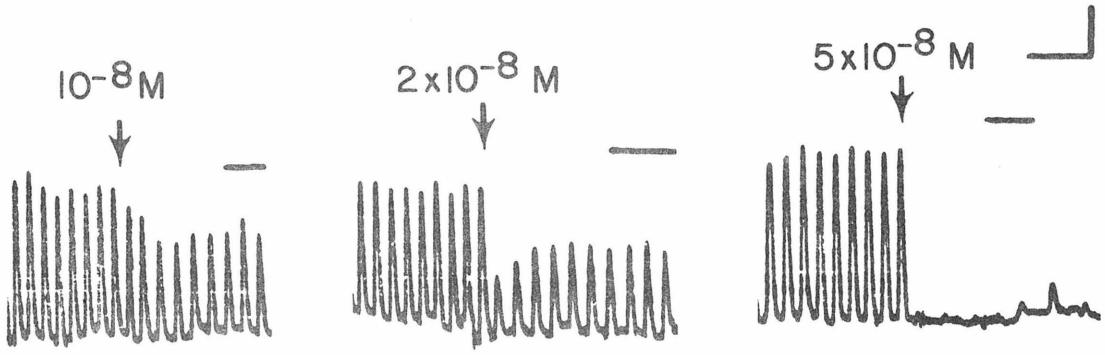


Figure 31

Figure 32. Antagonism by MXC of action of conusine and ACh on clam heart.

Antagonism of the clam heart response to conusine and ACh by MXC at very low concentrations is shown on two different hearts. As in the case of BZQ and TEA, antagonism by MXC was very difficult to reverse. In the two traces shown, considerable blockade of both conusine and ACh action remained after 1 hr and after 40 min, respectively, of extensive rinsing following application of 10^{-7} M MXC and 5×10^{-7} M MXC, respectively. Calibration: 1 min, 0.1 g.

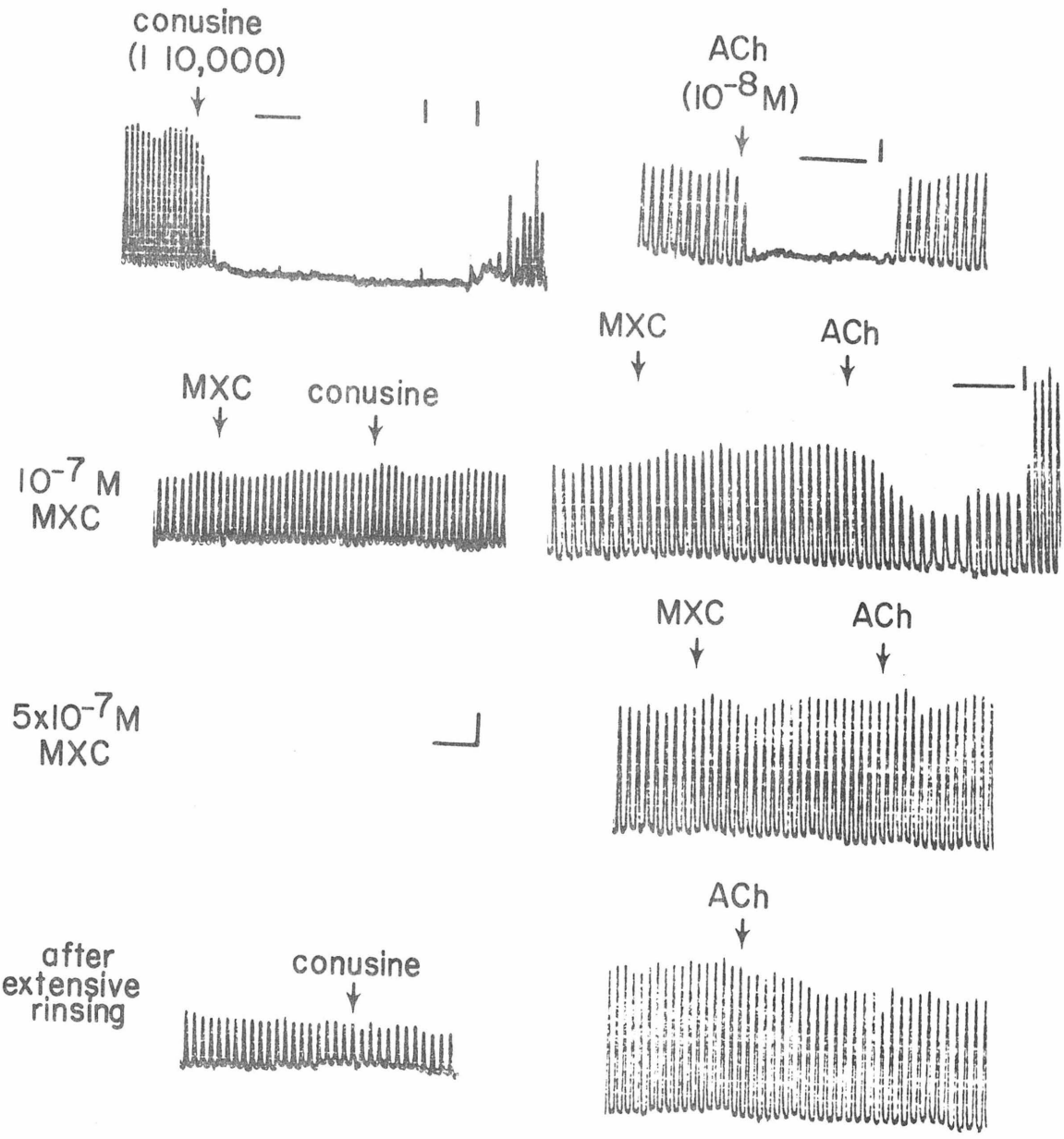

conusine
(1 10,000)
ACh
(10^{-8} M)
MXC
conusine
MXC
ACh
10^{-7} M
MXC
MXC
ACh
$5x10^{-7}$M
MXC
ACh
after
extensive
rinsing
conusine

Figure 32

Figure 33. Antagonism by PTMA of action of conusine and ACh on clam heart.

The effects of increasing concentrations of PTMA on the action of conusine and ACh in two different hearts are shown. PTMA is a far less effective antagonist than BZQ or MXQ and appears to be somewhat less effective than TEA. (In other hearts tested, PTMA at concentrations of 10^{-4} M and 5×10^{-5} M was able to completely block conusine and ACh action. In these cases, PTMA exerted marked excitatory effects of its own.) Antagonism by PTMA was found to be very difficult to reverse. Calibration: 1 min, 0.1 g.

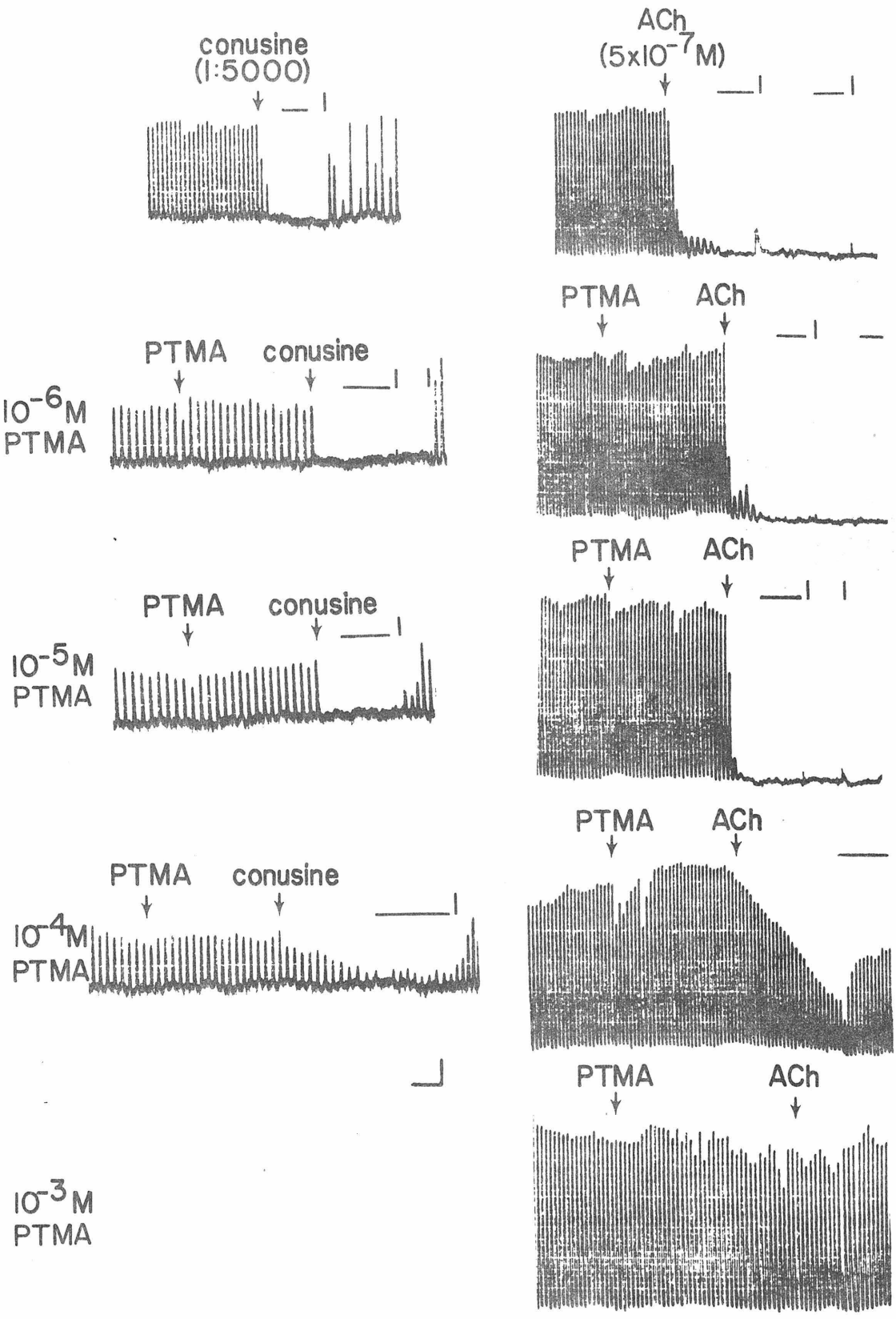

conusine
(1:5000)
ACh
(5x10⁻⁷M)
PTMA
conusine
10⁻⁶M
PTMA
PTMA
ACh
PTMA
conusine
10⁻⁵M
PTMA
PTMA
ACh
PTMA
conusine
10⁻⁴M
PTMA
PTMA
ACh
10⁻³M
PTMA
PTMA
ACh

Figure 33

Figure 34. Effect of Bgt on conusine action on clam heart.

The effect of a series of concentrations of Bgt on the response to conusine of a single heart is shown. For each concentration, the heart's response to the given concentration of conusine was tested, the heart was bathed with Bgt for 30 min, and its response to the same concentration of conusine was again tested. Unlike the case with ACh, Bgt shows virtually no blockade of conusine even at a concentration of 5×10^{-4} M Bgt. Calibration: 1 min, 0.1 g.

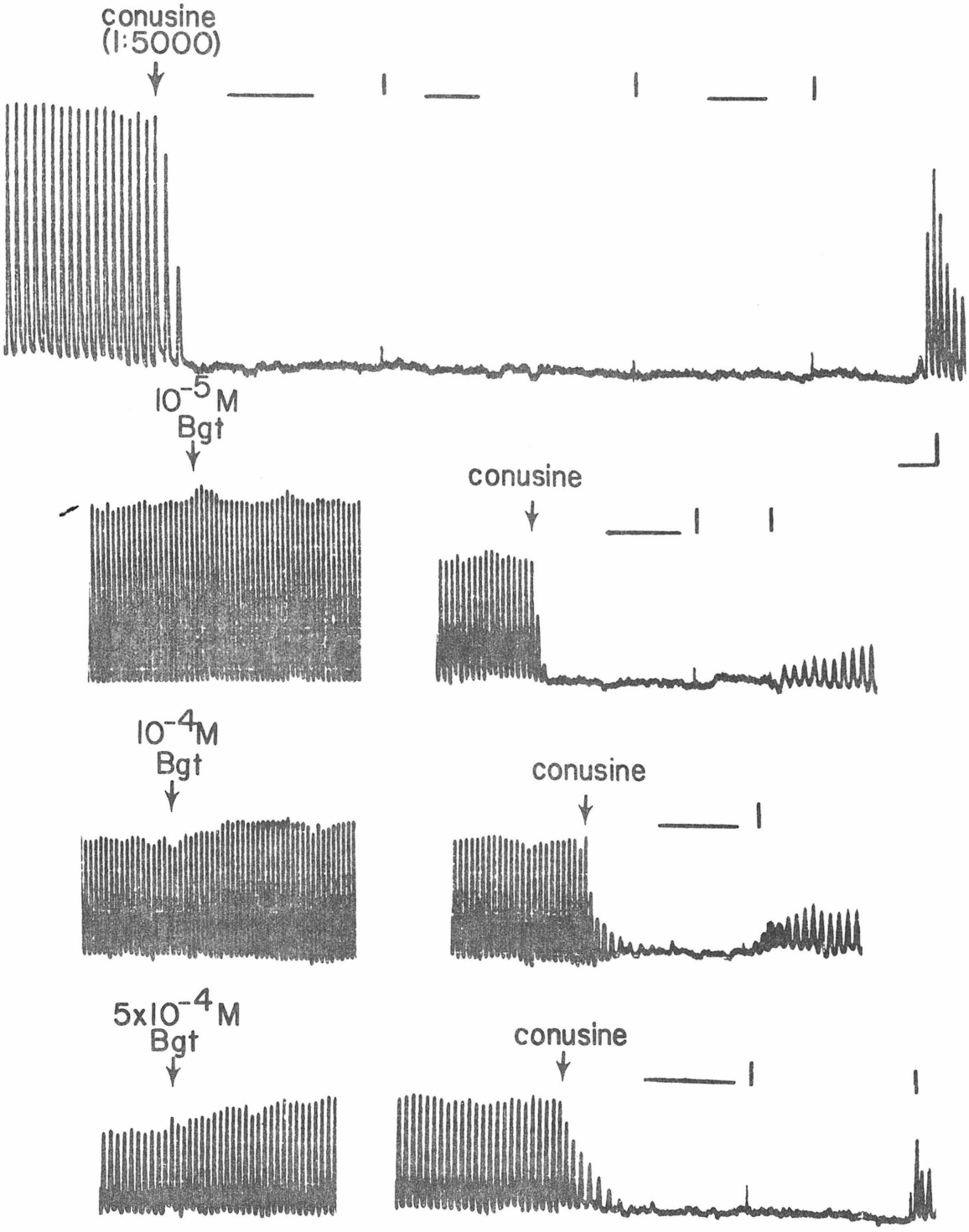

conusine
(1:5000)
10^{-5} M
Bgt
conusine
10^{-4}M
Bgt
conusine
$5x10^{-4}$ M
Bgt
conusine

Figure 34

Figure 35. Effect of Bgt on ACh action on clam heart.

The effect of a series of concentrations of Bgt on the ACh response of four different hearts is shown. In each case, the heart's response to a given concentration of ACh was tested, the heart was bathed with Bgt for 30 min, and the response to the same concentration of ACh was again tested. The higher concentrations of Bgt appear to inhibit ACh action, but this blockade is totally reversible after one to several rinses. Calibration: 1 min, 0.1 g.

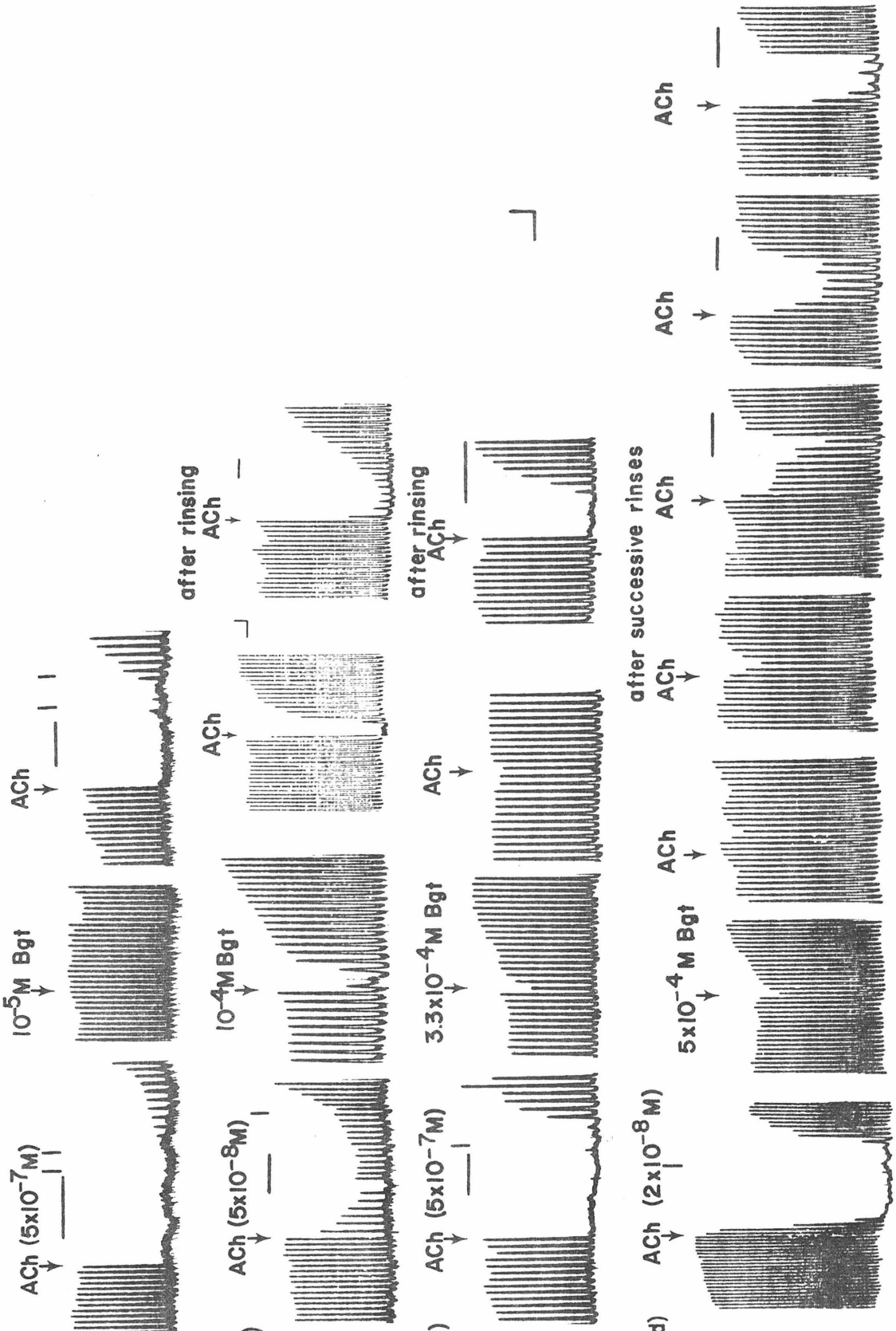

(a)
ACh (5x10⁻⁷M)
10⁻⁵M Bgt
ACh
(b)
ACh (5x10⁻⁸M)
10⁻⁴M Bgt
ACh
after rinsing
ACh
(c)
ACh (5x10⁻⁷M)
3.3x10⁻⁴M Bgt
ACh
after rinsing
ACh
(d)
ACh (2x10⁻⁸M)
5x10⁻⁴M Bgt
ACh
after successive rinses
ACh
ACh

Figure 35

Figure 36. Effect of changes in external K^+ on ACh action on clam heart.

The effects of K^+-free and $2 \times K^+$- (20 mM) ASW on the responses of two different clam hearts to increasing concentrations of ACh are shown. For each concentration of ACh, the response was recorded several times in normal ASW, the bath was flushed with 70 ml of K^+-altered ASW, and the response to ACh was again recorded several times. The bath was then rinsed with 70 ml of normal ASW and the next concentration of ACh assayed. The traces show no significant differences in the effectiveness of a given concentration of ACh in either K^+-free or $2 \times K^+$-ASW. Calibration: 30 sec, 0.1 g.

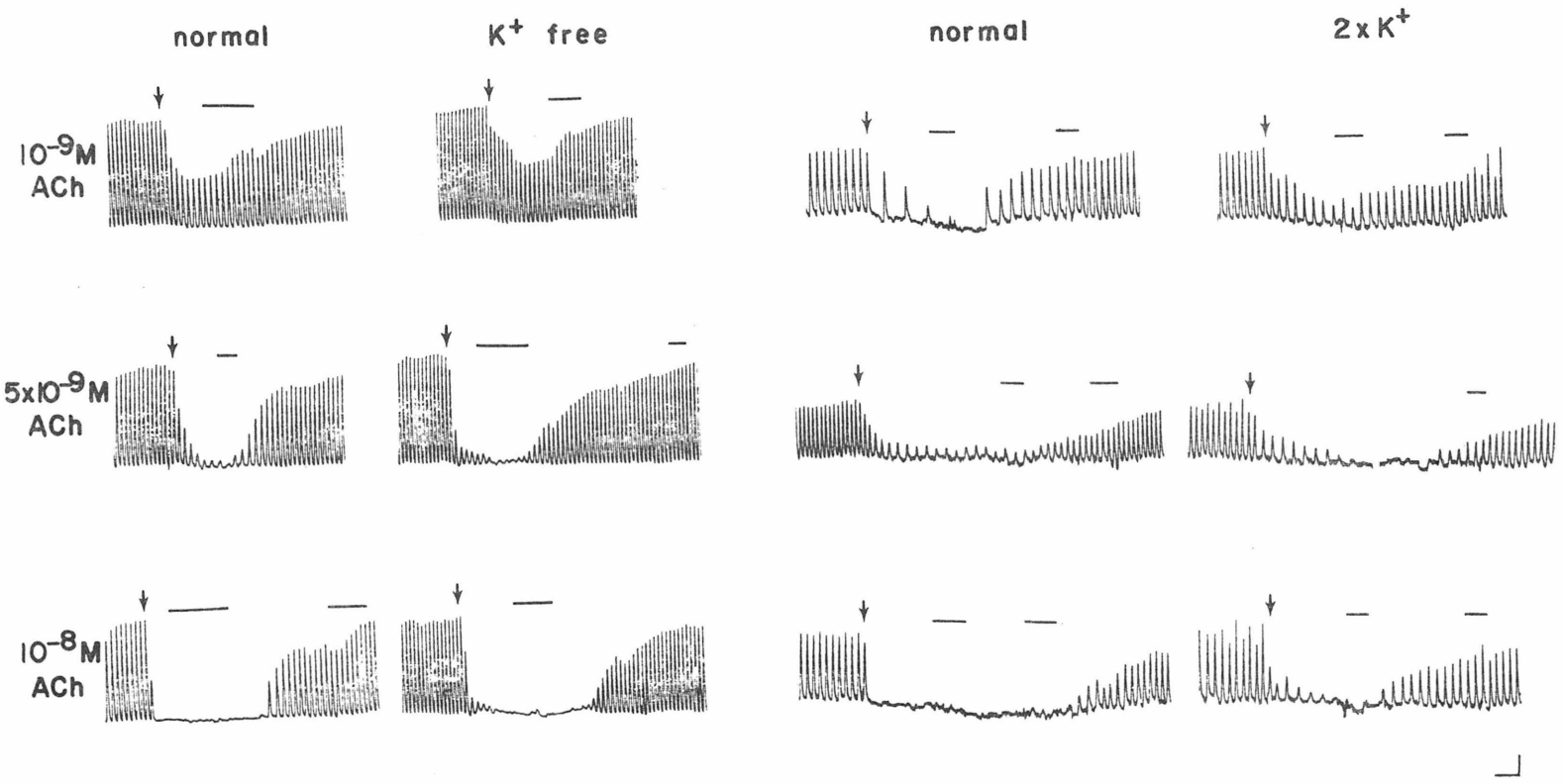


Figure 36

Figure 37. Effect of Cl^--free ASW on ACh action on clam heart.

The effect of Cl^--free ASW on the response of a single clam heart to increasing concentrations of ACh is shown. For each concentration of ACh, the response was recorded in normal ASW, the bath was perfused with 70 ml of Cl^--free ASW and the ACh response in Cl^--free ASW was recorded, and the bath solution was then returned to normal with a 70 ml perfusion of normal ASW and the ACh response was again recorded. The traces indicate an apparent increase in ACh effectiveness, by a factor of roughly two, in Cl^--free ASW. This result was observed in all three hearts tested. Calibration: 0.5 min, 0.1 g.

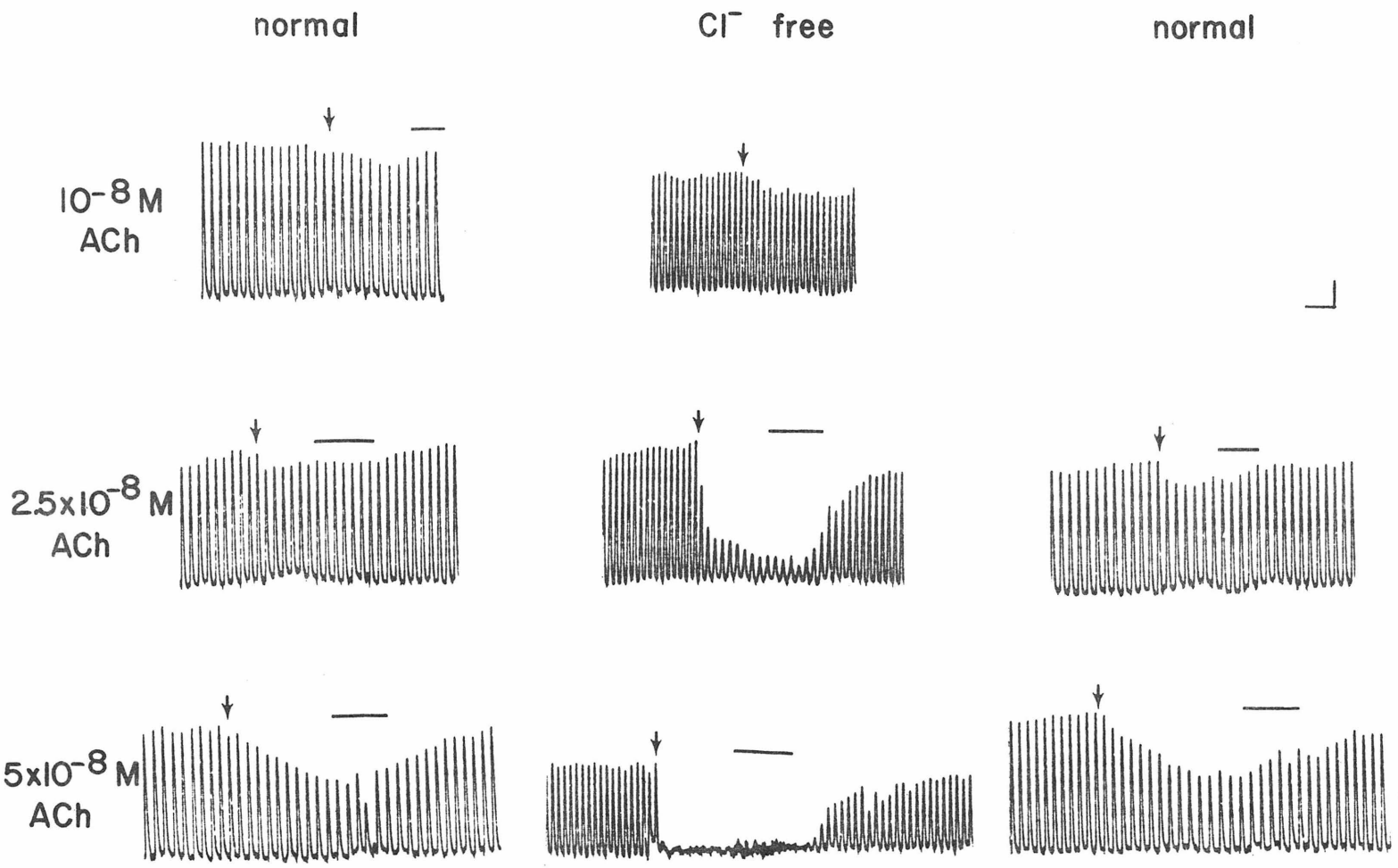


Figure 37

		A	B	C
silica gel TLC	conusine (sg TLC 1)	0		
	ACh	0		
	BZQ	0		
	flaxedil	0		
	MXC	.13		
	dTC	.36		
	(arecoline	.87)		
paper chromatogram	conusine (sg TLC 1)	.26	.27, .13	.63
	ACh	.66	.50, .29	.83
	BZQ	.74, .69	.69, .29	.86
	flaxedil		.42	.88, .76
	MXC	.88	.82	.94
	dTC	.66	.67	.87
cellulose TLC (a)	conusine (G-50 III)	.47, .66	.33, .44	.46, .59
	conusine (sg TLC 1)	.52, .66	.38, .49	.54, .65
	ACh	.71	.41, .53	.74
	BZQ	.82	.66	.74
	flaxedil	.79	.44	.81
	MXC	.80	.82	.84
(b)	conusine (cell TLC 4)	.54	.26	.59
	conusine (sg TLC 1)	.54, .69	.24, .49	.60, .70
	ACh	.70	.41	.75
	TMA	.81	.42	.83
	neurine	.74	.40	.78
	homarine	.64	.38	.68
	N-methylpyridinium	.77	.44	.79

Table 1. R_f values of conusine and some quaternary ammonium compounds. Each group represents values for compounds developed on the same chromatogram. The solvent systems A, B, and C are described in Figure 9.

AGONISTS		Mercenaria heart	Aplysia neurons (58)
conusine	+++	$6x10^{-2}$ - $6x10^{-3}$ ducts /ml	+++
arecoline	+++	$5x10^{-8}$ - $5x10^{-7}$M	+++
carbamylcholine	+++	$4x10^{-7}$ - $4x10^{-6}$M (101)	+
methacholine	++	$5x10^{-5}$ - $5x10^{-4}$M (101)	0
nicotine	+	10^{-4}M	0
TMA	+	$5x10^{-4}$M (101)	0
muscarine	+	$5x10^{-4}$M	0
decamethonium	0	(70)	0
pilocarpine	0	(102)	0
ANTAGONISTS			
BZQ	+++	$5x10^{-7}$-10^{-6}M (70, 103)	+
MXC	+++	$5x10^{-7}$-10^{-6}M	++
TEA	++	$5x10^{-4}$-10^{-4}M (70, 102)	++
PTMA	++	$5x10^{-5}$-$5x10^{-4}$M	+++
dTC	0	(102)	0
dihydro-β-erythroidine	0	(70)	0
hexamethonium	0		0
flaxedil	0		0
atropine	0	(102)	0

	Mercenaria heart	Aplysia neurons
+++	acts at very low concentrations	absolutely specific for slow hyperpolarizing response
++	acts at moderate concentrations	relatively specific for slow hyperpolarizing response
+	acts only at very high concentrations	acts also on other two cholinergic receptors at similar conc.
0	no effect at any concentration	no effect at any concentration

Table 2. Effectiveness of cholinergic agonists and antagonists on mercenaria heart and on the slow hyperpolarizing cholinergic response of Aplysia neurons. Concentrations listed are those sufficient to give complete inhibition of clam heart (diastolic arrest) or complete antagonism.

DISCUSSION

1. Comments on the Purification and Chemical Structure of Conusine

The three stage purification procedure which was adopted was very suitable for a preliminary purification effort, since all of the steps provided some secondary means, other than bioassay, of locating the conusine. In the G-50 fractionation, once conusine activity was shown to coincide with a large peak in A_{280}, the position of conusine in the G-50 elution profile could be routinely monitored by absorption at 280 nm rather than by the much more tedious and sample-consuming method of bioassay. Similarly, in the silica gel and cellulose TLCs, once the position of conusine activity had been shown by bioassay to coincide with an iodine-stainable spot of characteristic R_f, conusine could be followed by iodine staining. However, it appears that the latter two steps gave unexpectedly low recoveries of conusine, as low as 20% for both silica gel and cellulose semi-preparative TLC. Recoveries in these steps, especially silica gel TLC, seem to be improved by extracting conusine from the adsorbent with aqueous ammonium acetate (20 mM to 1.0 M) rather than water or methanol, but such an extraction procedure also extracts proportionately more of the organic binder and other contaminants.

Using the information that has been obtained on the biochemical properties of conusine, it may be possible now to devise a better purification procedure for the isolation of conusine, i.e., one which gives better recoveries and accumulates fewer contaminants than TLC on semi-preparative plates. It is understandable that Sephadex ion

exchange resins did not prove suitable for the purification of a molecule as small and highly charged as conusine. The stronger ion exchange resins of the Dowex series are more suited for chromatography of this type of compound. The fractionation of conusine on such resins might be conveniently monitored by analytical TLC of fraction aliquots, with bioassay of those fractions which contain iodine or DPA-stainable spots.

If conusine does indeed prove to be a quaternary ammonium compound, extraction with DPA may be a very quick method of separating conusine from non-quaternary components. This method, which has been shown to be much more suitable for isolation of very small amounts of quaternary ammonium compounds than the standard procedure of precipitation as reineckate or enneaiodide (55), was developed by Eksborg and Persson (21) for the spectrophotometric analysis of ACh, and has been modified for quantitative isolation of choline derivatives and other quaternary ammonium compounds by Jenden and associates (D. J. Jenden, personal communication). The extraction with DPA involves the formation between DPA and the quaternary nitrogen of an ion-pair which is extractable into an organic phase, leaving behind those components soluble only in aqueous phase. With the addition of acid, this ion-pair dissociates, allowing the quaternary nitrogen compound to be extracted back into aqueous phase and recovered by lyophilization or evaporation. Such an extraction procedure can recover around 90%-95% of the material (D. J. Jenden, personal communication).

Although no specific chemical structure can be proposed as the structure of conusine, a number of compounds have been excluded (see

Table I), including arecoline, whose physiological activity appears thus far to be indistinguishable from that of conusine. The selection of better purification techniques may facilitate the determination of the chemical structure of conusine, by allowing greater quantities of purified material to be obtained from the same number of Conus. Ultimately, if conusine proves to be a component of the venoms of other Conus species (see below), it may be preferable to isolate conusine from a species of larger size (which is to say, from practically any species other than C. californicus).

2. Physiological Role of Conusine

Although a physiological activity for the purified component conusine has been clearly demonstrated in the invertebrates Aplysia and Mercenaria, its role in the activity of the whole venom of C. californicus cannot be concluded from the above experiments. Some interesting speculations can be made, however. As mentioned in the Introduction, Whysner and Saunders (113) have reported that the whole venom appears to paralyze other gastropods, the natural prey of C. californicus. Such a paralytic action could result either from the blockade of excitatory neuromuscular synapses or the activation of inhibitory ones. Unfortunately very little is known about neuromuscular transmission in molluscs. It is generally accepted that ACh is probably the inhibitory transmitter and 5-HT the excitatory transmitter in the cardioregulatory nerves of Mercenaria, and perhaps in a number of other molluscs (41). The only molluscan muscle other than the heart

on which sufficient work has been done to name a probable transmitter is the anterior byssus retractor muscle of the mussel Mytilus edulis, for which ACh is probably the excitatory transmitter (see 33 for review). No neuromuscular inhibition other than cardiac inhibition has been observed in molluscs (33), leaving open the possibility that there is no peripheral inhibition of the body musculature, but certainly indicating that we know nothing about the mechanism of peripheral inhibition in molluscs, if it does exist.

It is conceivable that conusine may be capable of activating a putative peripheral inhibitory cholinergic receptor in molluscs in an analogous fashion to its action on Aplysia neurons or on clam heart. In such a hypothesis, conusine would be mimicking the action of ACh and, because of its insusceptibility to esterase hydrolysis, would produce an exaggerated or prolonged cholinergic effect. A model for such a hypothesis is provided by the action of arecoline on parasitic flukes. Arecoline is commonly used in veterinary medicine (and for human treatment in some parts of the world) as a vermifuge (63). Pharmacological evidence suggests that ACh is probably an inhibitory neuromuscular transmitter in the parasitic flukes Schistosoma mansoni and Fasciola hepatica, and possibly in other trematodes (8). Arecoline produces a paralysis of the body musculature in these worms (especially the oral suckers), which is blocked by atropine and appears to involve muscarinic-type receptors. Conusine may act in an analogous fashion in molluscs. By assaying purified conusine on isolated molluscan muscle preparations, one should be able to test this hypothesis.

Many of the symptoms of C. californicus venom poisoning in vertebrates which were observed by Whysner and Saunders (113; and

by other investigators for other Conus species; see 37, 89), such as lacrimation, sweating, increased salivation, and miosis indicate parasympathomimetic action of the venom (63), while hypothermia, convulsions, and blood pressure alterations indicate the activation of cholinergic receptors in the CNS (61). Arecoline injected in sufficiently high concentrations intravenously produces these same parasympathomimetic and central cholinergic symptoms in vertebrates (61). It is possible that at sufficiently high concentrations conusine could, by reaction with vertebrate cholinergic receptors, be responsible, at least in part, for the parasympathomimetic and central cholinergic effects of Conus envenomation.

Whysner and Saunders (113) have also shown that C. californicus whole venom contains one or more principles which can apparently paralyze vertebrate skeletal muscle. Their work indicated that the LD_{50} of the whole venom in mice was ~1.6 ducts/kg, i.e., 5% of the venom of one snail was sufficient to paralyze the respiratory musculature of a 30 g mouse. In the Results (see Figure 18) it was shown that from 1% to 10% of the conusine of one snail was strongly inhibitory to the clam heart. Purified conusine showed no activity on the frog rectus abdominis, which like the rat diaphragm is a nicotinic muscle, at concentrations which were 10 times that effective on the clam heart, and thus of about the same order of magnitude as the LD_{50} reported by Whysner and Saunders in mice. These preliminary experiments should be extended, however, before any conclusions as to the action of conusine on vertebrate skeletal musculature is made.

There are several precedents for the toxic action of small molecular weight, quaternary ammonium compounds derived from marine gastropods. Tetramethylammonium (tetramine) appears to be the main active principle in the salivary poison of the marine snail Neptunea arthritica (3), a carnivorous scavenger. The physiological role of the tetramine in the salivary glands has not been determined, but it may serve as a passive defense against predation. Consumption of the snail in Japan has led to many reported poisonings, with symptoms including headache, dizziness, nausea, and visual disturbances (3). The pharmacological actions of tetramine in mice include both antagonism of neuromuscular cholinergic activity, with resultant paralysis and respiratory failure, and parasympathomimetic stimulation of peripheral organs, evidenced by increased lacrimation, salivation, miosis, etc. (3).

A second relevant example of potent small molecular weight cholinergic poisons is the choline ester urocanylcholine (murexine), found in the hypobranchial glands of marine snails of the genus Murex. Urocanylcholine acts as both a ganglionic cholinergic agonist and a strong neuromuscular antagonist (27), causing a depolarization of the muscle followed by cholinergic blockade. Whittaker (111, 112) has also described two other choline esters, with exactly the same actions in vertebrates as urocanylcholine, in the hypobranchial glands of the marine snails Buccinum undatum and Thais floridana. Here again, the physiological roles of these components have not been determined.

Since the physiological role of conusine is not definitely established, it is not possible to predict whether it should be present in the

venoms of other Conus and if so, which species. It is conceivable that conusine could be a metabolite or could function mainly in intracellular osmoregulation and ion balance, as probably do the quaternary ammonium compounds homarine, γ-butyrobetaine, and N-methyl-pyridinium (J. H. Welsh, personal communication) which are found in the venom of at least two Conus species (68). If this is the type of role conusine plays, then it may well be generally present in Conus venoms. If, on the other hand, it is a major toxic principle, it may be specific for one particular type of prey, such as gastropods or polychaete worms, and may be present only in other molluscivorous or vermivorous cone snails.

3. Cholinergic Receptors in the Clam Heart

In the experiments performed and described above, there was no pharmacological indication that more than one type of cholinergic receptor is present in Mercenaria cardiac cells. That is, when the inhibition of ACh was blocked by BZQ, TEA, or PTMA, there was no detectable residual effect, either excitatory or inhibitory, of applied ACh. However, as noted in the Introduction, bath application of drugs can mask the presence of multiple receptors. In addition, in this case the phenomenon being monitored is not the actual change in membrane potential but the resultant muscle contraction which is several steps removed. There could possibly be more than one phase to the cholinergic response which is not detectable by this secondary assay. Welsh and Taub (108) have reported that very low concentrations of ACh, below the threshold of inhibition, occasionally have an excitatory

effect on the heart. This suggests that there may be a second type of receptor present. As was described in the Introduction, a number of other bivalve hearts have been shown to have biphasic responses to ACh--an inhibition which is blocked by BZQ and an excitation which is blocked by dTC (36). It appears very likely that many of these preparations will prove to possess the presence of both the cholinergic receptor mediating a slow hyperpolarization and the cholinergic D receptor.

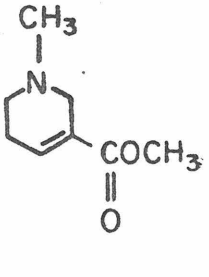
The experiments described above suggest very strongly the pharmacological equivalency of the cholinergic receptor in clam heart with the cholinergic receptor mediating the slow hyperpolarization in the medial cells of the Aplysia pleural ganglion. Definite proof of this equivalency awaits intracellular recording from single clam heart cells which can be shown to exhibit a slow hyperpolarization in response to iontophoretically applied ACh (and to presynaptic nervous stimulation) which is blocked by the right antagonists and mimicked by the right agonists.

This work which has been done so far is not conclusive enough to suggest equivalency of the ionic mechanisms underlying the cholinergic response in the clam heart and the slow hyperpolarizing cholinergic response in Aplysia. It would be premature to assume that, since the latter response is the result of an increase in K^+ permeability, then the cholinergic response in clam heart is also mediated by K^+. The pharmacological specificity of a receptor has not been shown to necessarily determine the ionophore to which it is coupled. [For example,

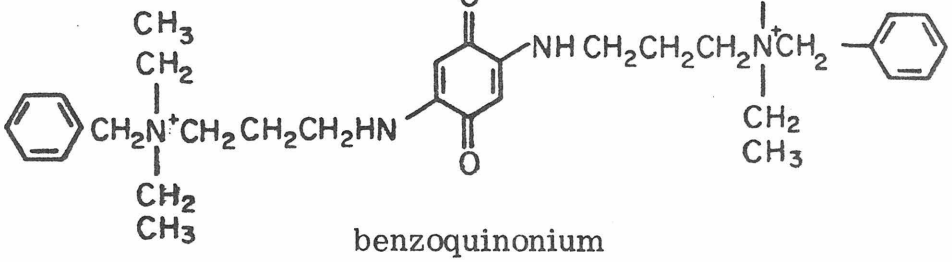
muscarinic receptors in vertebrate heart are coupled to K^+ channels and mediate a hyperpolarization (39, 44, 98, 99) while in smooth muscle they mediate a depolarization which is probably due to an increase in permeability to Ca^{++} or Na^+ or both (9).] Here again, intracellular studies on clam cardiac cells should be able to provide more definitive information regarding the ionic mechanism of the cholinergic response than can an assay of muscle contraction, which is susceptible to ionic effects at any of several stages between the actual permeability change and muscle contraction.

APPENDIX 1

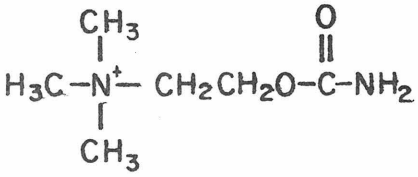
STRUCTURE OF SOME CHOLINERGIC COMPOUNDS



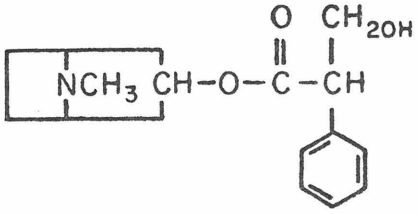
arecoline



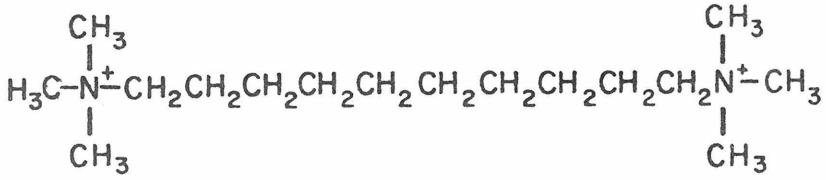
benzoquinonium



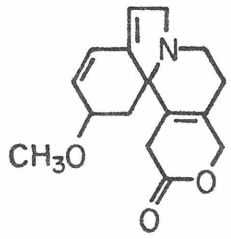
carbamylcholine



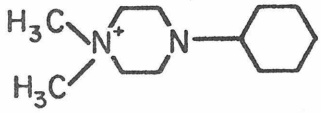
atropine



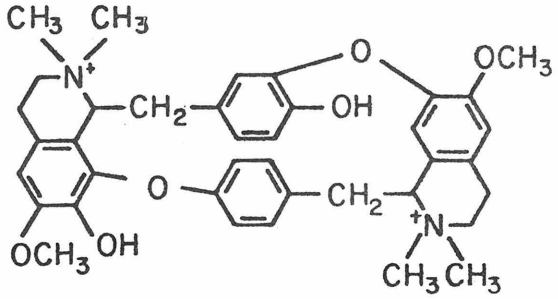
decamethonium



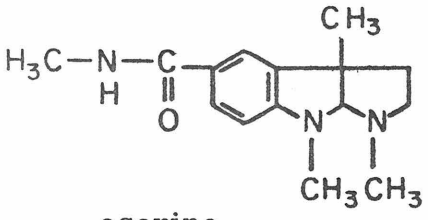
dihydro-β-erythroidine



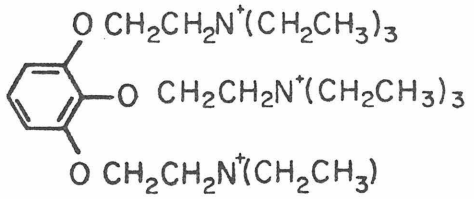
dimethylphenylpiperazinium



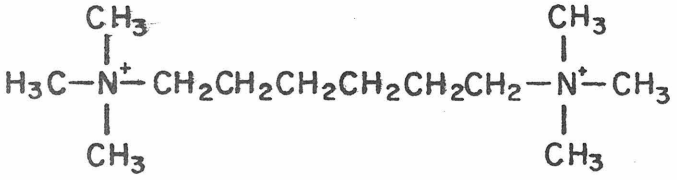
d-tubocurarine



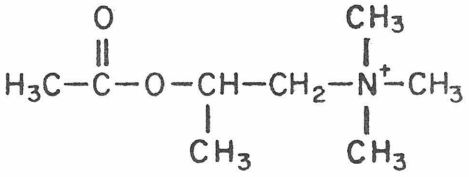
eserine
(physostigmine)



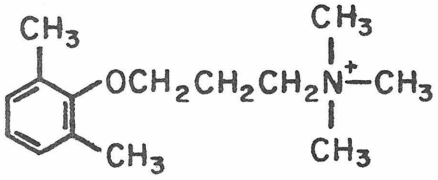
flaxedil
(gallamine)



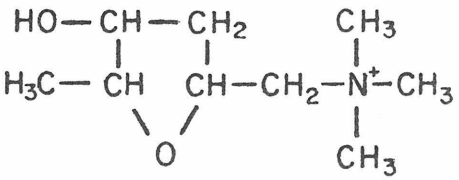
hexamethonium



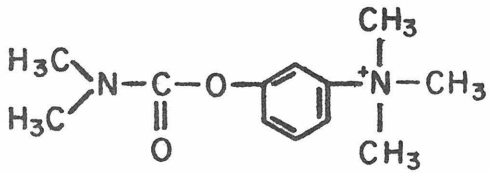
methacholine
(acetyl-β-methylcholine)



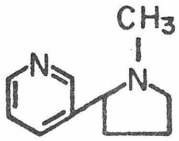
methylxylocholine



muscarine

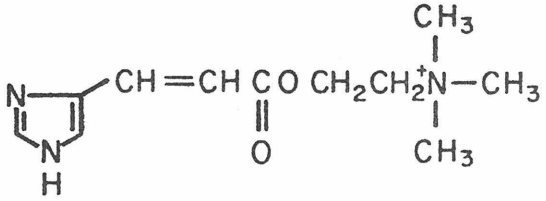


neostigmine
(prostigmine)



nicotine

pilocarpine



urocanylcholine
(murexine)

APPENDIX 2

PARTIAL CLASSIFICATION OF THE ANIMAL KINGDOM

The following partial classification (from Marshall and Williams, 77) outlines the relationships among the organisms referred to in this thesis.

Phylum PLATYHELMINTHES
- Class: Digenea (also known as trematodes; flukes)
  - Genuses: Schistosoma, Fasciata

Phylum ANNELIDA
- Class: Polychaeta (marine worms)
- Class: Hirudinea (leeches)

Phylum MOLLUSCA
- Class: Gastropoda
  - Subclass: Prosobranchia
    - Genuses: Conus, Buccinum, Murex, Neptunea (marine snails)
  - Subclass: Opistobranchia
    - Genuses: Aplysia, Navanax
  - Subclass: Pulmonata
    - Genuses: Helix, Onchidium, Cryptomphallus (land or fresh water snails)
- Class: Bivalvia (Pelecypoda)
  - Order: Anisomaria
    - Genuses: Mytilus (mussel), Ostrea (oyster)
  - Order: Heterodonta
    - Genuses: Mercenaria, Protothaca, Mya (clams)

Phylum CHORDATA
- Subphylum Vertebrata

REFERENCES

1. Albuquerque, E. X., M. Sasa, B. P. Avner, J. W. Daly. 1971. Possible site of action of batrachotoxin. Nature N. B. 234, 93-95.

2. Albuquerque, E. X., J. E. Warnick, R. M. Sansone. 1971. The pharmacology of batrachotoxin. II. Effect on electrical properties of mammalian nerve and skeletal muscle membrane. J. Pharm. exp. Therap. 176, 511-528.

3. Asano, M., M. Itoh. 1960. Salivary poison of a marine gastropod, Neptunea arthritica Bernardi, and the seasonal variation of its toxicity. Ann. N. Y. Acad. Sci. 90, 674-690.

4. Ascher, P. 1972. Inhibitory and excitatory effects of dopamine on Aplysia neurones. J. Physiol. (London) 225, 173-209.

5. Ascher, P., J. S. Kehoe. 1975. Amine and amino acid receptors in gastropod neurons. In Handbook of Psychopharmacology. Eds. L. L. Iversen, S. D. Iversen, S. H. Snyder. Plenum Publishing Corporation, New York. In press.

6. Blankenship, J. E., H. Wachtel, E. R. Kandel. 1971. Ionic mechanisms of excitatory, inhibitory, and dual synaptic actions mediated by an identified interneuron in abdominal ganglion of Aplysia. J. Neurophysiol. 34, 76-92.

7. Block, R. J., E. L. Durrum, G. Zweig. 1958. A Manual of Paper Chromatography and Paper Electrophoresis, 2nd ed. Academic Press, New York.

8. Bueding, E., J. Bennett. 1972. Neurotransmitters in trematodes. In Comparative Biochemistry of Parasites. Ed. H. Van den Bossche. Academic Press, New York. pp. 95-99.

9. Burnstock, G., M. E. Holman, C. L. Prosser. 1963. Electrophysiology of smooth muscle. Physiol. Rev. 43, 482-527.

10. Chiarandini, D. J., H. M. Gerschenfeld. 1967. Ionic mechanism of cholinergic inhibition in molluscan neurons. Science 156, 1595-1596.

11. Chiarandini, D. J., E. Stefani, H. M. Gerschenfeld. 1967. Ionic mechanisms of cholinergic excitation in molluscan neurons. Science 156, 1597-1599.

12. Chiba, S., Y. Nagawa. 1971. Effects of nereistoxin and its derivatives on the spinal cord and motor nerve terminals. Japan. J. Pharmacol. 21, 175-184.

13. Cleland, W. W. 1964. Dithiothreitol, a new protective reagent for SH groups. Biochem. 3, 480-482.

14. Clench, W. J., U. Kondo. 1943. The poison cone shell. Am. J. Trop. Med. 23, 105-120.

15. Cottrell, G. A. 1970. Direct postsynaptic responses to stimulation of serotonin-containing neurones. Nature 225, 1060-1062.

16. Cottrell, G. A. 1971. Synaptic connections made by two serotonin-containing neurons in the snail (Helix pomatia) brain. Experientia 27, 813-815.

17. Cottrell, G. A., N. N. Osborne. 1970. Subcellular localization of serotonin in an identified serotonin-containing neurone. Nature

225, 470-472.

18. Cottrell, G. A., B. M. Twarog. 1972. Active factors in the venom duct of Conus californicus. Brit. J. Pharm. 44, 365-366.

19. Dawson, R. M. C., D. C. Elliott, W. H. Elliott, K. M. Jones, eds. 1969. Data for Biochemical Research, 2nd ed. Oxford University Press, New York.

20. Eccles, J. C. 1966. The ionic mechanisms of excitatory and inhibitory synaptic action. Ann. N. Y. Acad. Sci. 137, 473-494.

21. Eksborg, S., B. A. Persson. 1971. Photometric determination of acetylcholine in rat brain after selective isolation by ion pair extraction and microcolumn separation. Acta Pharm. Suecica 8, 205-216.

22. Ellmann, G. L., K. D. Courtney, V. Andres, Jr., R.-M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

23. Endean, R., J. Izatt. 1965. Pharmacological study of the venom of the gastropod Conus magus. Toxicon 8, 81-93.

24. Endean, R., J. Izatt, D. McColm. 1967. The venom of the piscivorous gastropod Conus striatus. In Animal Toxins. Eds. F. E. Russell, P. R. Saunders. Pergamon Press, New York. pp. 137-144.

25. Endean, R., C. Rudkin. 1963. Studies of the venoms of some Conidae. Toxicon 1, 49-64.

26. Endean, R., C. Rudkin. 1965. Further Studies of the venoms

of Conidae. Toxicon 2, 225-249.

27. Erspamer, V., A. Glasser. 1957. The pharmacological actions of murexine (urocanylcholine). Brit. J. Pharmacol. 12, 176-184.

28. Fiske, C. H., Y. SubbaRow. 1925. The colorimetric determination of phosphorous. J. Biol. Chem. 66, 375-400.

29. Florey, E. 1967. The clam heart bioassay for acetylcholine. Comp. Biochem. Physiol. 20, 365-377.

30. Florey, E., H. J. Merwin. 1961. Inhibition in molluscan hearts and the role of acetylcholine. In Nervous Inhibition. Ed. E. Florey. Pergamon Press, New York. pp. 136-143.

31. Freeman, S. E., R. J. Turner. 1972. A myotoxin secreted by some piscivorous Conus species. Brit. J. Pharmacol. 46,

32. Gardner, D., E. R. Kandel. 1972. Diphasic postsynaptic potential: a chemical synapse capable of mediating conjoint excitation and inhibition. Science 176, 675-678.

33. Gerschenfeld, H. M. 1973. Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. Physiol. Rev. 53, 1-119.

34. Gerschenfeld, H. M., D. Paupardin-Tritsch. 1974. Ionic mechanisms and receptor properties underlying the responses of molluscan neurones to 5-hydroxytryptamine. J. Physiol. (London) 243, 427-456.

35. Greenberg, M. J. 1965. A compendium of responses of bivalve hearts to acetylcholine. Comp. Biochem. Physiol. 14, 513-539.

36. Greenberg, M. J. 1970. A comparison of acetylcholine structure-activity relations on the hearts of bivalve molluscs. Comp. Biochem. Physiol. 33, 259-294.

37. Halstead, B. W. 1965. Poisonous and Venomous Animals of the World. vol. I, Invertebrates. U. S. Govt. Printing Office, Washington, D. C.

38. Hanna, G. D., A. M. Strong. 1949. West American Mollusks of the Genus Conus. Proc. Calif. Acad. Sci. 9, 247-322.

39. Harris, E. J., O. F. Hutter. 1956. The action of acetylcholine on the movements of potassium ions in the sinus venosus of the heart. J. Physiol. (London) 133, 58-59P.

40. Hermitte, L. C. D. 1946. Venomous marine molluscs of the genus Conus. Roy. Soc. Trop. Med. Hyg. 39, 485-512.

41. Hill, R. B., J. H. Welsh. 1966. Heart, circulation and blood cells. In Physiology of Mollusca, vol. II. Eds. K. M. Wilbur, C. M. Yonge. Academic Press, New York. pp. 125-174.

42. Hille, B. 1970. Ionic channels in nerve membranes. Prog. Biophys. Mol. Biol. 21, 1-32.

43. Hinegardner, R. T. 1958. The venom apparatus of the cone shell. Hawaii Med. J. 17, 533-536.

44. Hutter, O. F., W. Trautwein. 1956. Vagal and sympathetic effects on the pacemaker fibres in the sinus venosus of the heart. J. Gen. Physiol. 39, 715-733.

45. Innes, I. R., M. Nickerson. 1970. Drugs inhibiting the action of acetylcholine on structures innervated by postganglionic

parasympathetic nerves (antimuscarinic or atropinic drugs). In The Pharmacological Basis of Therapeutics, 4th ed. Eds. L. S. Goodman, A. Gilman. The MacMillan Company, New York. pp. 524-548.

46. Irisawa, H., A. Irisawa, N. Shigeto. 1969. Effects of Na^+ and Ca^{++} on the spontaneous excitation of the bivalve heart muscle. In Comparative Physiology of the Heart: Current Trends. Ed. F. McCann. Experientia, Supp. 15. Burkhauser Verlag, Basel. pp. 176-191.

47. Irisawa, H., M. Kobayashi, T. Matsubayashi. 1961. Action potentials of oyster myocardium. Jap. J. Physiol. 11, 162-168.

48. Irisawa, H., M. Kobayashi. 1964. On the spontaneous activities of oyster myocardium caused by several inorganic ions in sucrose solution. Jap. J. Physiol. 14, 165-176.

49. Irisawa, H., A. Noma, R. Ueda. 1968. Effect of calcium on the spontaneous activities of the oyster myocardium in sodium free solution. Jap. J. Physiol. 18, 157-168.

50. Irisawa, H., N. Shigeto, M. Otani. 1967. Effect of Na^+ and Ca^{++} on the excitation of the Mytilus (bivalve) heart muscle. Comp. Biochem. Physiol. 23, 199-212.

51. Irisawa, H., N. Shigeto, I. Seyama. 1967. Dual effect of acetylcholine on bivalve heart. The Physiologist 10, 210.

52. Jenden, D. J., L. B. Campbell. 1971. Measurement of choline esters. In Methods of Biochemical Analysis. Supplemental Volume: Analysis of Biogenic Amines and their Related

Enzymes. Ed. D. Glick. Interscience Publishers, New York. pp. 183-216.

53. Jenden, D. J., I. Hanin. 1974. Gas chromatographic microestimation of choline and acetylcholine after N-demethylation by sodium benzenethiolate. In Choline and Acetylcholine: Handbook of Chemical Assay Methods. Ed. I. Hanin. Raven Press, New York. pp. 135-150.

54. Jullien, H., D. Vincent, M. Bouchet, M. Vuillet. 1938. Observations sur l'acetylcholine et la choline-esterase du coeur des mollusques. Ann. Phys. et Phys. Biol. 14, 567-574.

55. Karlén, B., G. Lundgren, I. Nordgren, B. Holmstedt. 1974. Ion-pair extraction and gas-phase analysis of acetylcholine and choline. In Choline and Acetylcholine: Handbook of Chemical Assay Methods. Ed. I. Hanin. Raven Press, New York. pp. 163-180.

56. Karlin, A. 1973. Molecular interactions of the acetylcholine receptor. Fed. Proc. 32, 1847-1853.

57. Kehoe, J. S. 1972. Ionic mechanisms of a two-component cholinergic inhibition in Aplysia neurones. J. Physiol. (London) 225, 85-114.

58. Kehoe, J. S. 1972. Three acetylcholine receptors in Aplysia neurones. J. Physiol. (London) 225, 115-146.

59. Kehoe, J. S. 1972. The physiological role of three acetylcholine receptors in synaptic transmission. J. Physiol. (London) 225, 147-172.

60. Kehoe, J. S., P. Ascher. 1970. Reevaluation of the synaptic activation of an electrogenic sodium pump. Nature 225, 820-823.

61. Koelle, G. B. 1970. Neurohumoral transmission and the autonomic nervous system. In The Pharmacological Basis of Therapeutics, 4th ed. Eds. L. S. Goodman, A. Gilman. The MacMillan Company, New York. pp. 402-441.

62. Koelle, G. B. 1970. Anticholinesterase agents. In The Pharmacological Basis of Therapeutics, 4th ed. Eds. L. S. Goodman, A. Gilman. The MacMillan Company, New York. pp. 442-465.

63. Koelle, G. B. 1970. Parasympathomimetic agents. In The Pharmacological Basis of Therapeutics, 4th ed. Eds. L. S. Goodman, A. Gilman. The MacMillan Company, New York. pp. 466-477.

64. Koelle, G. B. 1970. Neuromuscular blocking agents. In The Pharmacological Basis of Therapeutics, 4th ed. Eds. L. S. Goodman, A. Gilman. The MacMillan Company, New York. pp. 601-619.

65. Kohn, A. J. 1956. Piscivorous gastropods of the genus Conus. Proc. Nat. Acad. Sci. USA 42, 168-171.

66. Kohn, A. J. 1959. The ecology of Conus in Hawaii. Ecol. Monogr. 29, 47-90.

67. Kohn, A. J. 1963. Venomous marine snails of the genus Conus. In Venomous and Poisonous Animals and Noxious Plants of the Pacific Region. Eds. H. L. Keegan, W. V. MacFarlane. The MacMillan Company, New York. pp. 83-95.

68. Kohn, A. J., P. R. Saunders, S. Weiner. 1960. Preliminary studies on the venom of the marine snail Conus. Ann. N. Y. Acad. Sci. 90, 706-725.

69. Krijgsman, B. J., G. A. Divaris. 1955. Contractile and pacemaker mechanisms of the heart of molluscs. Biol. Rev. 30, 1-39.

70. Lee, C.-Y. 1972. Chemistry and pharmacology of polypeptide toxins in snake venoms. Annual Rev. Pharmacol. 12, 265-286.

71. Lee, C.-Y., C.-C. Chang, C. Su, Y. W. Chen. 1962. The toxicity and thermostability of Formosan snake venoms. J. Formosan Med. Assoc. 61, 239-244.

72. Levitan, H., L. Tauc. 1972. Acetylcholine receptors: topographic distribution and pharmacological properties of two receptor types on a single molluscan neurone. J. Physiol. (London) 222, 537-558.

73. Loveland, R. E. 1963. 5-hydroxytryptamine, the probable mediator of excitation in the heart of Mercenaria (Venus) mercenaria. Comp. Biochem. Physiol. 9, 95-104.

74. Lowry, O. H., U. S. Rosebrough, A. L. Farr, R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

75. Luduena, F. P., T. G. Brown, Jr. 1952. Mytolon and related compounds as antagonists of acetylcholine on the heart of Venus mercenaria. J. Pharm. exp. Ther. 105, 232-239.

76. McMichael, D. F. 1971. Mollusks- classification, distribution,

venom apparatus and venoms, symptomatology of stings. In Venomous Animals and their Venoms, vol. III, Invertebrates. Eds. W. Bücherl, E. E. Buckley. Academic Press, New York. pp. 373-393.

77. Marshall, A. J., W. D. Williams, eds. 1972. Textbook of Zoology, vol. I, Invertebrates, 7th ed. American Elsevier Publishing Company, Incorporated, New York.

78. Moore, J. W., T. Narahashi. 1967. Tetrodotoxin's highly selective blockage of an ionic channel. Fed. Proc. 26, 1655-1663.

79. Nagawa, Y., Y. Saji, S. Chiba, T. Yuu. 1971. Neuromuscular blocking actions of nereistoxin and its derivatives and antagonism by sulfhydryl compounds. Japan. J. Pharm. 21, 185-197.

80. Narahashi, T. 1974. Chemicals as tools in the study of excitable membranes. Physiol. Rev. 54, 813-889.

81. Narahashi, Y. 1970. Pronase. In Methods in Enzymology, vol. XIX, Proteolytic Enzymes. Eds. G. E. Perlmann, L. Lorand. Academic Press, New York. pp. 651-664.

82. Oomura, U., H. Ooyama, M. Sawada. 1965. Ionic basis of the effect of acetylcholine on D and H neurons. Proc. Intern. Congr. Physiol. Sci. 23rd, Tokyo. Abstr. 389.

83. Pilgrim, R. L. C. 1954. The action of acetylcholine on the hearts of lamellibranch molluscs. J. Physiol. 125, 208-214.

84. Prosser, C. L. 1940. Acetylcholine and nervous inhibition in the heart of Venus mercenaria. Biol. Bull. 78, 92-102.

85. Prosser, C. L., F. A. Brown. 1965. Comparative Animal

Physiology, 3rd ed. Saunders Publishing Company, Philadelphia.

86. Reed, K., R. Vandlen, J. Bode, J. Duguid, M. A. Raftery. 1975. Characterization of acetylcholine receptor-rich and acetylcholinesterase-rich membrane particles from Torpedo californica electroplax. Arch. Biochem. Biophys. In press.

87. Roop, T., M. J. Greenberg. 1967. Acetylcholinesterase activity in Crassostrea virginica and Mercenaria mercenaria. Amer. Zool. 7, 737-738.

88. Russell, F. E. 1965. Marine toxins and venomous and poisonous marine animals. Adv. Mar. Biol. 3, 255-384.

89. Russell, F. E. 1967. Comparative pharmacology of some animal toxins. Fed. Proc. 26, 1206-1224.

90. Sato, M., G. Austin, H. Yai, J. Maruhashi. 1968. The ionic permeability changes during acetylcholine-induced responses of Aplysia ganglion cells. J. Gen. Physiol. 51, 321-325.

91. Saunders, P. R., F. Wolfson. 1961. Food and feeding behavior in Conus californicus Hinds. 1844. The Veliger 3, 73-76.

92. Schmidt, J., M. A. Raftery. In preparation. Biochemical characterization of acetylcholine receptors.

93. Shain, W., L. A. Greene, D. O. Carpenter, A. J. Sytkowski, Z. Vogel. 1974. Aplysia acetylcholine receptors: blockade by and binding of α-bungarotoxin. Brain Res. 72, 225-240.

94. Smith, C. C., D. Glick. 1939. Some observations on cholinesterase in invertebrates. Biol. Bull. 77, 321.

95. Szczepaniak, A. C. 1974. Effect of αbungarotoxin and Dendroaspis

neurotoxins on acetylcholine responses of snail neurones. J. Physiol. (London) 241, 55-56P.

96. Szczepaniak, A. C., G. A. Cottrell. 1973. Biphasic action of glutamic acid and synaptic inhibition in an identified serotinin-containing neurone. Nature N. B. 241, 62-64.

97. Tauc, L. 1967. Transmission in invertebrate and vertebrate ganglia. Physiol. Rev. 47, 521-593.

98. Trautwein, W., J. Dudel. 1958. Zum Mechanismus der Membranwirkung des Acetylcholin an der Herzmuskelfaser. Pflügers Arch. ges. Physiol. 266, 324-334.

99. Trautwein, W., S. W. Kuffler, C. Edwards. 1956. Changes in membrane characteristics of heart muscle during inhibition. J. Gen. Physiol. 40, 135-145.

100. Tu, A. T., B.-S. Hong, T. N. Solie. 1971. Characterization and chemical modifications of toxins isolated from the venoms of the sea snake, Laticauda semifasciata from Philippines. Biochem. 10, 1295-1304.

101. Turner, R. J., S. E. Freeman. 1974. Factors affecting the muscle depolarization due to Conus achatinus toxin. Toxicon 12, 49-55.

102. Volle, R. L., G. B. Koelle. 1970. Ganglionic stimulating and blocking agents. In The Pharmacological Basis of Therapeutics, 4th ed. Eds. L. S. Goodman, A. Gilman. The MacMillan Company, New York. pp. 585-600.

103. Wachtel, H., E. R. Kandel. 1967. A direct synaptic connection

mediating both excitation and inhibition. Science 158, 1206-1208.

104.Wachtel, H., E. R. Kandel. 1971. Conversion of synaptic excitation to inhibition at a dual chemical synapse. J. Neurophysiol. 34, 56-68.

105.Wait, R. B. 1943. The action of acetylcholine on the isolated heart of Venus mercenaria. Biol. Bull. 85, 79-85.

106.Welsh, J. H. 1953. Excitation of the heart of Venus mercenaria. Arch. exper. Path. u. Pharmakol. 219, 23-29.

107.Welsh, J. H., A. G. Slocombe. 1952. The mechanism of action of acetylcholine on the Venus heart. Biol. Bull. 102, 48-57.

108.Welsh, J. H., R. Taub. 1948. The action of choline and related compounds on the heart of Venus mercenaria. Biol. Bull. 95, 346-353.

109.Welsh, J. H., R. Taub. 1950. Structure-activity relationships of acetylcholine and quaternary ammonium ions. J. Pharm. exp. Therap. 99, 334-342.

110.Welsh, J. H., R. Taub. 1953. The action of acetylcholine antagonists on the heart of Venus mercenaria. Brit. J. Pharmacol. 8, 327-333.

111.Whittaker, V. P. 1958. Acrylylcholine: a new naturally occurring pharmacologically active choline ester from Buccinum undatum. Biochem. Pharmacol. 1, 342-346.

112.Whittaker, V. P. 1960. Pharmacologically active choline esters in marine gastropods. Ann. N. Y. Acad. Sci. 90, 695-705.

113.Whysner, J. A., P. R. Saunders. 1963. Studies on the venom of

the marine snail Conus californicus. Toxicon 1, 113-122.

114.Whysner, J. A., P. R. Saunders. 1966. Purification of the lethal fraction of the venom of the marine snail Conus californicus. Toxicon 4, 177-181.

115.Whyte, J. M., R. Endean. 1962. Pharmacological investigation of the venoms of the marine snails Conus textile and Conus geographus. Toxicon 1, 25-31.

116.Worthington Enzymes Manual. 1969. Worthington Biochemical Corporation, Freehold, New Jersey.

117.Yang, C. C. 1967. The disulfide bonds of cobratoxin and their relationship to lethality. Biochim. Biophys. Acta 133, 346-355.

118.Zweig, G., J. Sharma, eds. 1972. Chemical Rubber Company Handbook of Chromatography, vol. II. Chemical Rubber Company Press, Cleveland, Ohio.