CHAPTER 5

DISCUSSION
Although it is clear that intercellular communication in the nervous system generally involves the release of chemical transmitter substances from nerve terminals, the knowledge about modulation of neurochemical transmission is still incomplete. However, in the past few years, several observations have been reported which suggest as to how the amount of transmitter released at nerve terminals may be modulated either by another transmitter/modulator released from another nerve terminal (Vizi and Knoll, 1971; Vizi, 1974a; Muscholl, 1973) or by an "auto-inhibition" mechanism wherein the transmitter released into the synaptic cleft may inhibit its own release (Langer, 1973, 1974; Stjarne, 1975; Starke et al, 1977; Starke, 1977; Westfall, 1977).

In recent years presynaptic mechanisms modulating the release of transmitter have been extensively investigated in the central nervous system and at peripheral sympathetic neurones (Langer, 1977; Starke, Taube and Borowski, 1977). Results are, however, less conclusive in respect to the presynaptic effects of acetylcholine and cholinomimetics at cholinergic synapses including the skeletal myoneural junction (Nistri, 1976a&b; Kilbinger, 1977a&b). In the present study efforts have been made to identify the role played by presynaptic muscarinic and prostaglandin receptors on the release of acetylcholine from rat phrenic nerve terminals and from the Auerbach's plexus of guinea-pig ileum.

The skeletal myoneural effects of oxotremorine, a tremorogenic and muscarinic agent, has been reported by several investigators. However, anomaly exists regarding oxotremorine's mechanism of action at this site. Cho, Haslett and Jenden (1962) and Levy and Michel-Ber
(1967) suggested a curare-like paralytic effect of oxotremorine at skeletal myoneural junction. Contrarily, Elmqvist and McIssac (1967) demonstrated a direct depolarization of the muscle end-plate in presence of oxotremorine. Ganguly and Chaudhuri (1970) suggested an indirect action of oxotremorine mediated through excess release of acetylcholine at this site. More recently, Field and Bowen (1978) have provided evidence for an involvement of subreceptor effect i.e. an effect at sites distal to the receptors in the action of oxotremorine at skeletomotor junction.

In the present investigation efforts were made to find out the exact mechanism of action of oxotremorine at skeletal myoneural junction. Employing the technique of "pharmacological denervation" the involvement of postsynaptic receptors in the action of oxotremorine at skeletal neuromuscular junction was eliminated in the present study. It was found that a paralytic dose of oxotremorine failed to inhibit the responses of directly stimulated rat diaphragm in presence of the acetylcholine synthesis inhibitor, hemicholinium-3, at a time when there was failure in neuromuscular transmission (see Results).

Having eliminated a postsynaptic site of action of oxotremorine at myoneural junction, the resting and electrically evoked release of acetylcholine from the phrenic nerve was measured in presence of oxotremorine with the view to substantiate the presynaptic effect of the agent at skeletomotor junction. In the present study it was found that incubation of a fasciculatory (2.5 μM) as well as a paralytic (10 μM) dose of oxotremorine increased massively the amount of released (spontaneous and electrically induced) acetylcholine from motor nerve ending as compared to control (see Results).
This finding suggests that both the initial muscle fasciculations and the subsequent neuromuscular blockade produced by oxotremorine may arise from a massive increase in the amount of acetylcholine released by the motor nerve impulse. A similar effect may occur in the central nervous system as suggested by Holmstedt and Lundgren (1966). Oxotremorine has been reported to raise the acetylcholine levels in the central nervous system (Pepeu, 1963; Holmstedt, Lundgren and Sundwall, 1963; Ganguly and Saha, 1969). While Szerb and Somogyi (1973) observed a reduced release of acetylcholine from isolated cerebral cortical slices of the rat, Guggenheimer and Levinger (1975) and Mistry (1976a) have reported that oxotremorine, increased spontaneous release of acetylcholine from cat and frog spinal cord respectively.

The observations made with oxotremorine at skeletal motor nerve terminal in the present study directly demonstrate that the neuromuscular effects of oxotremorine is mediated through excess release of acetylcholine from motor endings.

Oxotremorine and other muscarinic agonists have been found to increase the acetylcholine content in brain of rats and mice (Haubrich, Reid and Gillette, 1972; Szerb and Somogyi, 1973; Choi, Roch and Jenden, 1973; Saelens, Simke, Schuman and Allen, 1974; Trabucchi, Cheney, Hanin and Costa, 1975b; Racagni, Cheney, Trabucchi and Costa, 1975). Oxotremorine has also been found to reduce the conversion of labeled precursor into acetylcholine in mouse brain (Schuberth, Sparf and Sundwall, 1969; Trabucchi, Cheney, Hanin and Costa, 1975b). Since this reduced conversion does not compensate for the increased brain acetylcholine content it is
possible that the turnover rate of acetylcholine is decreased in
presence of oxotremorine (Cheney and Costa, 1977). However, with
the present experimental findings it is not possible to suggest
whether the increase in release of acetylcholine at myoneural junc-
tion caused by oxotremorine is due to increased synthesis or decreased
turnover rate of the transmitter.

The acetylcholine releasing action of oxotremorine is pro-
bably mediated through activation of presynaptic excitatory muscar-
nic receptors at motor nerve ending. Such a contention is further
supported by the observation that muscarine could produce a con-
centration-dependent increase of acetylcholine release from
phrenic nerve terminals and that in presence of atropine, the mus-
carinic antagonist, the increase in release of acetylcholine caused
by oxotremorine and muscarine could be prevented.

That the presynaptic muscarinic receptors in the motor nerve
terminals participate in the local regulation of acetylcholine
release is evidenced in the present study by inhibition of normal
evoked release in presence of atropine in the bathing medium at a
concentration which did not affect the phrenic nerve action poten-
tials. Although such an inhibition of transmitter release by atropine
from motor nerve ending has been reported (Hubbard and Wilson, 1970),
the exact mechanism of this inhibition remained obscure. The present
study raises the distinct possibility that endogenously liberated
acetylcholine from motor nerve endings causes activation of pre-
synaptic muscarinic receptors at this site leading to its own further
release. Recently evidence for such a presynaptic action of acetyl-
choline at the excitatory neuromuscular junction of the locust has
been demonstrated employing electrophysiological techniques (Fulton and Usnerwood, 1977).

The present observation that the acetylcholine releasing action of oxotremorine and muscarine is inhibited by dopamine and apomorphine and that this effect of dopamine and apomorphine in turn is prevented by pimozide, the dopamine receptor antagonist, suggest that presynaptic dopaminceptive sites, functionally antagonistic to excitatory muscarinic receptors, exist on motor nerve terminals. However, these presynaptic dopaminceptive sites do not appear to participate in the local regulation of acetylcholine release at skeletal myoneural junction. Such a contention is evidenced by the inability of pimozide to increase the evoked release of acetylcholine in control experiments, at a dose which completely prevented the inhibitory influence of exogenously administered dopamine and apomorphine on oxotremorine and muscarine-induced acetylcholine release. This is expected since, unlike sympathetic ganglia (Bjorklund, Cegrell, Flack, Ritzen and Rosengren, 1970), neither dopamine containing cells are known to be present at motor nerve endings nor dopamine is released by motor nerve stimulation. But in the light of these findings it seems plausible that systemic circulatory dopamine, more so in subjects receiving L-DOPA therapy, must modulate the acetylcholine release from motor nerve terminals, if not at the cholinergic synapses in general. Pharmacological implications of this presynaptic dopamine-action may be of therapeutic significance in L-DOPA medication since, a presynaptic cholinergic dominance at the neuromuscular junction and at the cholinergic junction between motor axon collaterals and Renshaw cells in the
spinal cord have been clearly implicated in the oxotremorine-model of Parkinsonism (Fackler, Ross, Cleveland and Haase, 1977; Cleveland, Ross, Ganguly, Kuschmierz and Haase, 1978; Ganguly, Nath, Ross and Vedasiromoni, 1978). Indeed, L-DOPA pretreatment was found to protect oxotremorine-induced tremor to an extent (Coward, Doggett and Sayers, 1977; Meyer-Lohmann, Hellweg, Hagneath and Benneck, 1972; Stadler, Lloyd, Gadea-Ciria and Bartholini, 1973) and to inhibit the antidromically activated spike discharges of single Renshaw cell (McGeer, McGeer, Grewaal and Singh, 1975). The regulation of cholinergic neurones by dopaminergic influence and vice versa is well documented in the central nervous system, where dopaminergic agonists inhibit the activity of cholinergic neurones as evidenced by a decrease in acetylcholine output (Sethy and Van Woert, 1973; Trabucchi, Cheney, Racagni and Costa, 1975a) and acetylcholine inhibits the evoked release of dopamine through muscarinic activation (Westfall, 1974a).

Functional roles have been proposed for presynaptic muscarinic receptors in the brain (Molenaar and Polak, 1970; Polak, 1971; Szerb and Somogyi, 1973; Bourdois, Mitchell, Somogyi and Szerb, 1974) and in the cholinergic innervation of gastrointestinal smooth muscle preparation (Vizi, 1974a), which appear to be at variance with the present observations at motor nerve terminals. In some of these studies oxotremorine caused inhibition of spontaneous as well as evoked release of acetylcholine and antagonised the facilitatory effect of atropine on acetylcholine release (Molenaar and Polak, 1970; Polak, 1971; Szerb and Somogyi, 1973; Vizi, 1974; Bourdois, Mitchell, Somogyi and Szerb, 1974; Guggenheimer and Levinger, 1975).
Contrarily, excess acetylcholine-release caused by oxotremorine at both central and peripheral sites are well documented (Gyorgy, Pfeifer & Kenyeres, 1970; Nistri, 1976a). Thus it becomes an open question as to whether the reported inhibition of acetylcholine release by oxotremorine is mediated through direct activation of inhibitory presynaptic muscarinic receptors or through release of some other inhibitory transmitter. Indeed, incubation with eserine, the anticholinesterase agent, inhibited acetylcholine release in the isolated cerebral cortical slices (Szerb and Somogyi, 1973; Bourdois, Mitchell, Somogyi and Szerb, 1974). The hypothesis of a negative feedback mechanism of acetylcholine mediated through presynaptic muscarinic receptors (Molenaar and Polak, 1970; Polak, 1971; Szerb and Somogyi, 1973; Bourdois, Mitchell, Somogyi and Szerb, 1974) appears not to be universally operative, especially in view of the observation that muscarine itself caused an increase in acetylcholine release from the guinea-pig myenteric plexus upon field stimulation (Kilbinger, 1977b) and in view of the present observations at motor nerve terminals.

The present findings demonstrate, for the first time to our knowledge, presence of presynaptic dopaminoceptive muscarinic receptors on motor nerve terminals and, in addition, indicate that acetylcholine and cholinomimetics can influence the release of transmitter from motor nerve endings. Existing evidence suggest that cyclic GMP mediates muscarinic cholinergic transmission in the ganglia leading to the production of the slow excitatory post-synaptic potential (Greengard and Kebabian, 1974). In the present study the role of cyclic nucleotides in the release of acetylcholine from motor nerve ending caused by muscarinic agents has not been investigated.
The role played by prostaglandins on acetylcholine release from motor nerve endings has, however, been investigated.

Prostaglandins have been found to be released along with acetylcholine during stimulation of cholinergic nerves (Ramwell et al, 1965; Laity, 1969). However, controversy exists regarding the exact role played by prostaglandins at cholinergic nerve endings. According to Wennmalm and Hedqvist (1971), prostaglandins play a role in acetylcholine liberation; they regulate cholinergic transmission by a negative feedback mechanism. On the other hand Ehrenpreis et al (1973) suggested that in guinea-pig ileum prostaglandins couple "cholinergic nerve terminals excitation with acetylcholine release". These authors observed that low concentrations of prostaglandin E_1 or E_2 reversed the inhibition of electrically-induced neurogenic contractions or isolated ileum caused by substances like morphine, prostaglandin antagonists and prostaglandin synthesis inhibitors (Ehrenpreis et al, 1973). Furthermore, indomethacin, the prostaglandin synthesis inhibitor, inhibited contractions of field stimulated longitudinal muscle of guinea-pig ileum (Ehrenpreis et al, 1973).

In the present study prostaglandin E_2 produced a concentration dependent increase in the amount of electrically evoked release of acetylcholine from phrenic nerve terminals. This observation is in agreement with the finding of Kadlec et al (1978) that the output of acetylcholine was increased by prostaglandin E_2.

Though indomethacin is commonly employed as a prostaglandin E synthesis inhibitor, it has already been reported that indomethacin also interact directly with the prostaglandin receptor system.
Controversy prevails regarding the physiological role played by prostaglandin E in acetylcholine liberation. On the basis of the observation that the output of acetylcholine was decreased by indomethacin and increased by prostaglandin E2, Kadlec et al. (1978) concluded that, physiologically, prostaglandin E2 may act as a modulator of cholinergic transmission. On the other hand, Hazra (1975) observed that indomethacin failed to alter significantly either the spontaneous acetylcholine output or the amount of acetylcholine released in response to field stimulation from Auerbach's plexus of guinea-pig ileum and suggested that prostaglandin E plays no physiological role in acetylcholine liberation from Auerbach's plexus of guinea-pig ileum (Hazra, 1975). Botting & Sulzman (1974) also could not find evidence for a prostaglandin E mediated negative feedback mechanism on acetylcholine release. The findings in the present study that indomethacin failed to affect the control release of acetylcholine suggest that prostaglandin E probably does not play any physiological role in acetylcholine release from motor nerve terminals as is the case from Auerbach's plexus of guinea-pig ileum (Hazra, 1975).

The prostaglandin E2 induced release of acetylcholine from motor nerve terminals is accomplished by activation of prostaglandin receptors and not by action on muscarinic or nicotinic cholinergic receptors on motor nerve terminals. Such a conclusion is reached because while indomethacin and 7-oxa-13-prostynoic acid, the prostaglandin receptor antagonist, inhibited the prostaglandin E2 induced increase of acetylcholine release from phrenic nerve terminals, atropine and hexamethonium failed to do so.
The present observations on the effect of oxotremorine, muscarine and atropine on acetylcholine release from Auerbach's plexus of guinea-pig ileum are in agreement with the observations of Cox and Hecker (1971), Vizi and Knoll (1972), Kilbinger and Wagner (1975) and Kilbinger (1977a&b), who have found that oxotremorine inhibited the release of acetylcholine from this preparation and that atropine antagonised the inhibitory effect of oxotremorine completely. Similar observations have been found in brain tissues in vivo (for references, see Jones, Guyenet, Cheramy, Gauchy and Glowinski, 1973) and in vitro (Bourdois, Mitchell, Somogyi and Szerb, 1974; Polak, 1971; Fosbraey and Johnson, 1980a), which led to the hypothesis that there is a local regulation of acetylcholine release via a negative feedback mechanism in the central cholinergic neurones (Polak, 1971). Furthermore, atropine and hyoscine, the muscarinic antagonists, have been found to facilitate the release of acetylcholine from brain tissues (for references, see Jones, Guyenet, Cheramy, Gauchy and Glowinski, 1973).

Evidence exist that exogenous acetylcholine can inhibit the output of transmitter acetylcholine by an action on presynaptic muscarine cholinergic receptors (Vizi, 1974a; Fosbraey and Johnson, 1978, 1980b). Kilbinger (1977a&b) has suggested that if the longitudinal muscle strip or ileum contain inhibitory muscarinic receptors which modulate acetylcholine release, the released endogenous acetylcholine should also activate these receptors and thus decrease its own further release.

In the present study, oxotremorine and muscarine facilitated the evoked acetylcholine release from Auerbach's plexus of guinea-pig
ileum at lower concentrations and at higher concentrations the agents inhibited acetylcholine release. While the latter effect has already been reported, the facilitatory effect has not been reported by anyone so far. This observation raises the possibility that two types of presynaptic muscarinic receptors, excitatory and inhibitory, are present in the Auerbach's plexus. While the excitatory muscarinic receptors are more sensitive to the effect of muscarinic agonists they are few in number. On the other hand the inhibitory presynaptic receptors are less sensitive to agonists but more in number. However, it appears that the presynaptic excitatory muscarinic receptors are more sensitive to the action of antagonists. Such a conclusion is based on the observation in the present study that atropine further increased the facilitation of acetylcholine release from Auerbach's plexus caused by lower concentrations of oxotremorine and muscarine. Furthermore, in presence of atropine the inhibition of acetylcholine induced by a higher concentration of oxotremorine and muscarine was changed to facilitation of release (Kilbinger, 1977a). It is interesting to mention in this connection the suggestion that the rate of acetylcholine release from the Auerbach's plexus depends on the collection period (Johnson, 1963; Vizi, 1974a). An inverse correlation existed between the rate of release and the duration of the collection period. When the acetylcholine from ileal segments was collected during 5, 10, 20 and 40 min periods, the total amount increased with the increase in collection period; during longer collection period the output of transmitter was reduced (Johnson, 1963).
It has been shown that dopamine inhibits the release of acetylcholine from the nerve terminals of Auerbach's plexus (Vizi, Ronai and Knoll, 1974b). In the present study dopamine (0.01 μM) failed to affect the normal release of acetylcholine from Auerbach's plexus as was observed by Paton and Vizi in 1969. Dopamine, however, inhibited the oxotremorine (0.025 μM) induced release of acetylcholine which was reversed by pimozide indicating that the inhibitory action of dopamine on oxotremorine-induced acetylcholine release is mediated via dopamine receptors. Pimozide did not affect the control (normal) and oxotremorine-induced release of acetylcholine from Auerbach's plexus. So it can be concluded that dopamine has no physiological role in acetylcholine release, from Auerbach's plexus of guinea-pig ileum.

In contrast to the findings of Hazra (1975), indomethacin and 7-oxa-13-prostynoic acid decreased both resting and stimulated output of acetylcholine from Auerbach's plexus of guinea-pig ileum. So prostaglandin E₂ may act physiologically as a modulator of acetylcholine release as was observed by Kadlec et al (1978).

There are increasing evidences in the literature showing that prostaglandins release acetylcholine from Auerbach's plexus of guinea-pig ileum (Wennmalm and Hedqvist, 1971; Kadlec et al, 1978). In the present study also prostaglandin E₂ released acetylcholine from Auerbach's plexus of guinea-pig ileum which was inhibited by indomethacin and 7-oxa-13-prostynoic acid but not by atropine.

Oxotremorine and nicotine, two agents commonly employed to produce Parkinson-like symptoms in experimental animals for the purpose of evaluating the anti-Parkinson potential of new agents,
have been reported to have profound action at skeletal myoneural junction (Hummel and Schulz, 1954a&b; Ganguly and Chaudhuri, 1970; Ganguly, 1976). Furthermore, classical anticholinergic anti-Parkinson drugs have been found to affect skeletal muscle and its transmission (Luilmann, Muscholl and Pract, 1959; Pract, 1959; Onuaguluchi and Lewis, 1963; Salako, 1970; Vedasiromoni and Ganguly, 1976) and a suggestion has already been made that this effect may contribute in the mechanism of their anti-Parkinson action (Onuaguluchi, 1964; Salako, 1970; Vedasiromoni and Ganguly, 1976). In the present study it has been found that cycrimine, benztropine and biperiden, three cholinolytic anti-Parkinson drugs, could inhibit the effects of oxotremorine and nicotine at the skeletal neuromuscular junction. Since these cholinolytic anti-Parkinson drugs effectively prevent the tremor induced by oxotremorine and nicotine, it seems plausible that one of the loci of antagonism between anti-Parkinson drugs and tremorogenic agents may be the cholinergic synapse at neuromuscular junction.

On the basis of the observation that section of the motor nerve failed to prevent the tremorogenic action of nicotine, Everett (1964) suggested that the effect of nicotine at skeletal myoneural junction has an important role to play in tremorogenesis by the agent. Recent studies indicate that the effect of oxotremorine at extra- and intra-fusal neuromuscular junction and at the junction between motor axon collaterals and Renshaw cells in the spinal cord should contribute in the genesis of tremor irrespective of the action of the agent on supraspinal structures (Ganguly and Chaudhuri, 1970; Ganguly et al, 1976; Fackler, et al, 1977; Ganguly et al, 1978).
Employing oxotremorine as a screening model the neuromuscular involvement has further been demonstrated in the anti-tremor action of propranolol (Ganguly, 1976) and TK 174 (1,1-bis(4-amino-phenyl)-propyl-(3)-amine), a substance which does not permeate the blood brain barrier (Leszkovszky and Tardos, 1971). The antagonism of the effect of oxotremorine and nicotine at skeletal myoneural junction by the three cholinolytic anti-Parkinson drugs, provides further support for the contention that the skeletomotor apparatus plays a major role in genesis and in prevention of tremor. It is possible that cycrimine, benztropine and biperiden inhibited the presynaptic effects of oxotremorine and nicotine at motor nerve terminals.

On the basis of the aforementioned possibility efforts were made to develop C_{10}Dichol, the peripheral acetylcholine synthesis inhibitor, as an anti-Parkinson agent. In the present study employing conventional evaluation procedures it has indeed been found that C_{10}Dichol possessed potent anti-Parkinson property.

This conclusion is based on the observations that C_{10}Dichol \( \text{decamethylene-bis-(hydroxyethyl)-dimethylammonium bromide} \) which impairs the synthesis of acetylcholine and does not pass the blood brain barrier could inhibit tremor induced by oxotremorine, physostigmine and nicotine as well as afford partial protection against physostigmine-induced lethality in mice (see Results). These tests are widely recognised as useful screening procedures for evaluation of anti-Parkinson drugs (Friedman and Everett, 1964; Jenden, 1968; Nose and Kojima, 1970).

Peripheral skeletomotor involvement in the "oxotremorine model" of Parkinsonism and a presynaptic muscarinic-dopaminergic
link in the motor nerve endings have been demonstrated in recent years (for references, see Field and Bowen, 1978). Like oxotremorine, the presynaptic effect of nicotine and physostigmine on motor nerve terminals mediated through excess acetylcholine are well documented (Barstad, 1962; Randic and Straughan, 1964; Chiou and Long, 1969; Chiou, 1973). It is thus possible to conclude that C_10 Dichol antagonised the neuromuscular and tremorogenic effects of the parkinsonimimetic agents, namely oxotremorine, nicotine and physostigmine by reducing the availability of acetylcholine at myoneural junction. Contrarily, neither the neuromuscular effects of arecoline and harmine which are mediated postsynaptically (Chaudhuri and Ganguly, 1974) nor their tremorogenic effects could be prevented by C_10 Dichol.

It may be mentioned in this connection that repository curare was used with success long back in 1959 to relieve tremor and rigidity in Parkinsonian patients (Berger, 1959), an observation that has remained obscure so far.

Protection by the peripherally acting drug, C_10 Dichol, against physostigmine-induced tremor and mortality demonstrates the involvement of peripheral sites in these effects of physostigmine. Thus the present results fail to confirm the suggestion that physostigmine-induced lethality is entirely central in origin (Nose and Kojima, 1970). It has earlier been suggested that nicotine tremor has both central and peripheral components (Everett et al., 1956) and that neuromuscular blocking agents prevent the same (Cahen and Lynes, 1951).

The effects of C_10 Dichol on the isolated frog rectus abdominis muscle and on the rat blood pressure indicate that neither the
postsynaptic nicotinic receptors of the skeletal muscle nor the peripheral muscarinic receptors are involved in the anti-tremor action of the drug. The mild anti-curare effect of C₁₀ Dichol on the isolated frog rectus muscle was presumably due to its weak anticholinesterase property (Barlow, 1955). Absence of any impairing influence of the maximum dose of C₁₀ Dichol used in the present study (0.08 mM) on indirect twitch responses of rat diaphragm coupled with the observation that the twitch tension of cat anterior tibialis muscle to indirect stimulation (0.1 Hz) was only marginally affected by an intravenous dose of 5 mg/kg of C₁₀ Dichol (Bowman and Hemsworth, 1965) suggest that the drug may not affect the normal neuromuscular function while producing its anti-tremor action.

The experiments with C₁₀ Dichol further confirm the hypothesis that cholinergic dominance at the extra- and intra-fusal neuromuscular junction is involved in oxotremorine-induced experimental parkinsonigenesis (for references, see Packler et al, 1977) and peripherally acting drugs may have some therapeutic value in Parkinsonism.