The pharmacology of the hemicholiniums

by

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INTRODUCTION

Acetylcholine (ACh), an important neurotransmitter in the central and peripheral nervous systems, is inactivated by the enzyme acetylcholinesterase (AChE). The role of AChE within the cholinergic system is, therefore, important and inhibition of this enzyme has profound pharmacological consequences. Organophosphates are irreversible inhibitors of AChE, and thus, in their presence ACh persists at the cleft of cholinergic synapses causing a dysfunction of neural pathways utilizing ACh as a transmitter (Taylor, 1980).

Choline, one of the products of ACh hydrolysis, is required for synthesis of ACh and reenters the presynaptic cell by an active high affinity sodium dependent choline transport system (Yamamura and Snyder, 1973). Hemicholinium-3 (HC-3) is a potent antagonist of this active transport, preventing ACh synthesis, and thus depleting presynaptic terminals of ACh (McIntosh and Collier, 1976). This action makes HC-3 a potential physiological antagonist to organophosphates.

While the pharmacological actions of HC-3 have been documented (for review see Bowman and Marshall, 1972), the bis-quaternary ammonium structure of this compound (and its consequent inability to cross the blood brain barrier) has limited its study as a putative antagonist to organophosphates, which do cross the blood brain barrier and have central effects such as respiratory depression. Harris, Stitcher and Heyl (1982), however, demonstrated that intraventricular administration of HC-3 protects against lethal doses of soman in both rats and rabbits, and proposed that the HC-3 effect was due to inhibition of ACh synthesis.

Since tertiary compounds are known to have better penetration through the blood brain barrier than quaternary compounds, a tertiary hemicholinium may prove useful as an antagonist to organophosphate poisoning. As no tertiary hemicholinium is at present commercially available, part of this thesis describes the synthesis of one such compound and its subsequent pharmacological evaluation.

One preparation that is suitable for pharmacological investigations into both organophosphates and hemicholiniums is the rat isolated phrenic nerve-diaphragm, which is a proven model for neuromuscular transmission (Bulbring, 1946). Bowman, Hemsworth and Rand (1967) demonstrated the presynaptic blockade of muscle twitch amplitude in this preparation and the cat anterior tibialis muscle by HC-3, and also showed antagonism of this blockade by choline.

Organophosphates (e.g.diisopropylphosphoroflouridate or DFP) have been shown to cause reversible depression of 60 Hz tetanic contraction amplitude, and enhance 0.2 Hz single twitch amplitude of the rat isolated phrenic nerve-diaphragm (Van der Meer and Meeter, 1956a,b), actions that have been correlated with a decrease in cholinesterase activity (Barstad, 1960). French, Wetherell and White (1979) using the same preparation showed that soman causes an irreversible block of 60 Hz tetanic contraction amplitude, an effect antagonized by pretreatment with pyridostigmine.

This thesis examines the pharmacological properties of HC-3 on the rat isolated phrenic nerve-diaphragm, and studies the antagonism by HC-3 of organophosphate (DFP and soman) induced depression of tetanic contraction amplitude and post-tetanic potentiation.

LITERATURE REVIEW

Pharmacology of the Hemicholiniums

Acetylcholine (ACh) is utilized as a neurotransmitter at several sites in the peripheral nervous system including the somatic neuromuscular junction, autonomic ganglia, and the parasympathetic neuroeffector junction. In the central nervous system, ACh is a known transmitter at spinal motoneuron axon collateral-Renshaw cells, and is a putative transmitter in several regions of the brain (for review see Cooper, Bloom and Roth, 1978).

ACh is synthesized at presynaptic terminals by choline acetyltransferase (CAT) and packaged into synaptic vesicles which fuse with the presynaptic membrane to cause release when the membrane is depolarized by a presynaptic action potential. The ACh transverses the synapse to bind with nicotinic receptors (ganglia, neuromuscular junction) or muscarinic receptors (parasympathetic neuroeffector junction). Inactivation of ACh is carried out by acetylcholinesterase (AChE), which hydrolyses the neurotransmitter to choline and acetate. Choline is actively taken up by the presynaptic terminal for resynthesis of ACh (for review of above see Kandel and Schwartz, 1981).

Several classes of drugs have been employed both clinically and experimentally to interfere with cholinergic transmission. Of these classes, neuromuscular blockers, ganglionic blockers, antimuscarinics, anticholinesterase agents, and inhibitors of ACh release had all been described by 1950 (Taylor, 1980). Until the discovery of hemicholiniums, no known drugs existed that could inhibit ACh synthesis per se.

The pharmacological evaluation of a series of α,α'quaternary ammonium salts synthesized from 4, 4'-bisacetophenone revealed three distinctly different mechanisms of action, dependent on the derivation of the salt (Long and Schueler, 1954; Schueler, 1955). Products derived from heterocyclic tertiary amines, were found to be potent anticholinesterase agents and were antagonized by atropine. Products derived from simple trialkyl tertiary amines exhibited the properties of rapidly acting neuromuscular blocking agents such as curare, and were antagonized by neostigmine and edrophonium. The third group of compounds derived from ethanol tertiary amines caused a delayed toxicity after intraperitoneal (i.p.) administration to mice, death occurring due to respiratory paralysis. This effect was antagonized by choline chloride. Compound number 3 in the series (α,α'dimethyl ethanolamino 4, 4'-bisacetophenone), was found to undergo spontaneous hemiacetal formation, and was thus named hemicholinium-3 or HC-3 (Schueler, 1955); the pharmacology of this compound, which was found to be the most toxic of the third group, has been studied in detail.

Schueler (1955) studied the toxicity of HC-3 in dogs, rabbits, guinea pigs, rats and mice and found that in all cases death resulted from a gradually-developing respiratory failure. Unlike most drugs, the toxic dose on a body-weight basis was greater, and the time to death longer, in larger animals. Schueler also found that in doses 100 times the LD_{50} an immediate neuromuscular paralysis could be induced. The delayed respiratory failure produced by HC-3 was poorly antagonized by anticholinesterase drugs, but pretreatment with choline protected against

the toxic effects (Schueler, 1955; Giovinco, 1957). Kase and Borison (1958) analyzed the effects of HC-3 by means of electrical stimulation of transections of the lower brain stem in unanaesthetized, pentobarbital anaesthetized and decerebrate cats. From their studies they concluded that the HC-3 respiratory paralyzant action was mainly due to depression of the respiratory regulatory mechanism, an action they thought took precedence over peripheral neuromuscular blockade. However, Longo (1959) found little change in phrenic action potentials recorded in cats and rabbits during full respiratory paralysis, a result more consistent with the known inability of quaternary ammonium compounds to penetrate the blood brain barrier.

Long and Schueler (1954) reported that HC-3 had no effect on the cat sciatic nerve-gastrocnemius muscle preparation when the nerve was stimulated at 0.1 Hz, however, 40-70 minutes after i.v. injection of HC-3, 1.0 Hz stimulation of the rabbit sciatic nerve resulted in a blockade of gastrocnemius muscle contraction (Reitzel and Long, 1959a). In addition it was found that HC-3 blockade of muscle contraction could be antagonized by choline chloride, or by removing the stimulus for ten to fifteen minutes (Reitzel and Long, 1959a).

Comparing a series of choline esters, Reitzel and Long (1959a) also concluded that the structure of choline was specific for antagonism of HC-3. Several esters of choline, including ACh were also effective antagonists but this was shown to be due to the choline liberated by their rapid hydrolysis, since the antagonism was prevented by tetraethylpyrophosphate (TEPP), an anticholinesterase. The specific antagonistic action

of choline and the frequency dependent blockade of muscle contraction suggested that HC-3 interferes with choline metabolism and thus causes a decreased synthesis of ACh.

McIntosh, Birks and Sastry (1956) added HC-3 to eserinized plasma perfusing the cats superior cervical ganglion, and found that after several minutes time lag, the rate of ACh release declined rapidly during repetitive stimulation. In addition, HC-3 diminished the rate of synthesis of ACh in minced mouse brain by 75%, but this action was much weaker in acetone-dried rat brain powder. As acetone destroys the integrity of membranes, McIntosh, Birks and Sastry (1956) proposed that HC-3 competes with choline for transport into nerve terminals by a specific carrier system. Gardiner (1961) confirmed this proposal when he found that HC-3 inhibited ACh synthesis in guinea pig brain homogenates, but had no effect on homogenates pretreated with ether, a procedure that disrupts membranes surrounding the CAT enzyme (Hebb and Smallman, 1956).

The inhibition by HC-3 of active choline secretory systems was demonstrated in avian kidney tubules (McIntosh, Birks and Sastry, 1956); and HC-3 was also shown to inhibit active uptake of choline by isolated perfused hearts (Buterbaugh and Spratt, 1968), and erythrocytes (Martin, 1969). It was not until the development of techniques utilizing isolated mammalian brain synaptosomes, that Marchbanks (1968) and Diamond and Kennedy (1969) demonstrated choline uptake by nervous tissue. However, in these investigations, very little synthesis of ACh was found to be associated with choline transported into synaptosomes. The reason for this was shown by Haga (1971) to be due to the fact that in the early

experiments relatively high concentrations of choline were used. When synaptosomes were exposed to low levels of choline, Haga (1971) observed that 50% of the radioactive choline taken up was converted to ACh. Based on this observation, Haga (1971) predicted the presence of two separate choline transport systems, one of which had a high affinity for choline.

Thereafter, Yamamura and Snyder (1973) and Haga and Noda (1973) demonstrated by kinetic analysis the presence of two choline transport systems in isolated rat brain synaptosomes. One was a low affinity system (Km = 90 μM) which was not energy-dependent, was not associated with an efficient synthesis of ACh, and did not parallel the regional distribution of CAT in the brain. Yamamura and Snyder (1973) showed that HC-3 was relatively ineffective in inhibiting the low affinity system, as at 100 μM concentration, it caused no greater reduction of choline synaptosomal accumulation than did neostigmine or atropine.

The other transport system had a high affinity for choline (Km = 4-8 μM), was highly sodium dependent, associated with an efficient synthesis of ACh, and had a regional distribution in brain which paralleled that of CAT. The dependency on sodium is strict as caesium and lithium do not affect choline uptake. Transport is optimal at low concentrations of potassium, and the system is also chloride dependent (Simon and Kuhar, 1976). Yamamura and Snyder (1973) also demonstrated that HC-3 (100 μM) reduced choline synaptosomal accumulation to 31% of control, HC-3 being the most active drug tested at this concentration. Other potent inhibitors of the system are bretylium and ACh (Haga and Noda, 1973). The potency of HC-3 and other bisalkonium compounds was confirmed by Barker and Mittag (1975),

who also found that potency in high affinity choline uptake inhibition was proportional to potency for ACh depletion. The high affinity choline uptake system is now regarded as the rate-limiting step in ACh synthesis.

The exact biochemical mechanism by which HC-3 inhibits ACh synthesis remains controversial, and the assumption that its presynaptic action is solely at the level of the plasma membrane may be an oversimplification. The effects of HC-3 on ACh turnover in muscles and ganglia are only slowly reversed by removing the drug from the medium (Cheymol, Bourillet and Ogura, 1962; Matthews, 1966). This is in contrast to the effects of postsynaptic blockers, which quickly dissappear when the medium is made drug-free. Some authors (McIntosh, 1961; Rodriguez de Lores Amaiz, Zieher and DeRobertis, 1970) have proposed that in addition to choline transport inhibition, HC-3 or its acetyl ester, may be stored and released as a false transmitter. Intracellular uptake of HC-3 in the rat striatum (Csillik, Haarstad and Knyihar, 1970) and canine caudate nucleus and hippocampus (Sellinger et al., 1969) has been demonstrated. However, Collier (1973) using the cat superior cervical ganglion preparation showed that although HC-3 accumulated intracellularly, it was not by the same mechanism as choline. Also, HC-3 did not replace ACh stoichiometrically, and could not be released by nerve stimulation.

In addition, although several studies have shown that HC-3 competitively inhibits CAT, and is itself acetylated _in vitro_ (Mann and Hebb, 1975; Bradshaw and Hemsworth, 1976) this has never been successfully demonstrated _in vivo_. As it is now considered that high affinity choline transport and ACh synthesis are kinetically coupled (Marchbanks and

Kessler, 1982), it is probable that HC-3 acts primarily by inhibiting choline transport (for review see Jope, 1979). One possibility that remains is that HC-3 may interfere with the transfer of ACh from the cytosol to synaptic vesicles, a process about which almost nothing is known.

The cholinergic actions of HC-3 are not entirely prejunctional. In large doses it produces an immediate respiratory paralysis which differs from the delayed effect of small doses in that it may be reversed by anticholinesterase drugs, but not by choline (Schueler, 1955). This suggests, as might be expected from its chemical structure, that large doses exert a postjunctional curare-like block at the neuromuscular junction, and this has been confirmed in experiments on isolated nerve muscle preparations (Reitzel and Long, 1959a; Bowman and Rand, 1961).

The postjunctional blocking action may contribute to a small extent to the paralysis produced by small doses, since, even before any transmission failure becomes evident (as judged by the amplitude of muscle twitch) some depression of responses to ACh and related agonists is observed (Bove and Haarstad, 1979). Any postjunctional blocking action must become relatively more important as transmitter output fails and normal safety margins in transmitter release become diminished (Paton and Waud, 1967).

In the sympathetic ganglia, the interonium distance in HC-3 is too great for effective combination with the postsynaptic receptors, and thus at this site, in contrast to the neuromuscular junction, transmission failure is almost exclusively due to presynaptic mechanisms (Bhatnager, Lam and McColl, 1965). In smooth muscle HC-3 has been shown to possess some

atropine-like action but this to too weak to contribute much to transmission failure (Bertolini, Greggia and Ferrari, 1967).

Intracellular recording techniques at the neuromuscular junction have confirmed that HC-3 possesses a postjunctional action. Martin and Orkand (1961a, b), using isolated frog extensor digitalis muscles, found that HC-3 decreased the amplitude of end plate potentials in response to nerve stimulation or iontophoretically applied ACh. This postsynaptic action was not antagonized by choline. Thies and Brooks (1961) confirmed this work using guinea pig serratus nerve-muscle preparations finding that the postsynaptic action of HC-3 is antagonized by eserine, but not by choline. In addition, the postsynaptic blockade of neuromuscular transmission by HC-3, unlike presynaptic blockade, is both immediate in onset, and is not dependent on frequency of stimulation.

The pharmacological effects of HC-3 have been studied in both peripheral and central cholinergic sites. In the periphery, HC-3 has been found to produce neuromuscular blockade in sciatic nerve-gastrocnemius muscle preparations of dogs, rabbits, and chickens (Reitzel and Long, 1959a), superior cervical ganglion-nictitating membrane of cats (Wilson and Long, 1959), and anterior tibialis-sciatic nerve of cats (Evans and Wilson, 1964).

In all of the above preparations, HC-3 at low doses is ineffective at stimulation frequencies below 1 Hz, and as stimulus frequency is increased, so the drug becomes more effective. At stimulation frequencies of 1 Hz or more a slowly developing decrease in muscle twitch amplitude is seen (Bowman and Marshall, 1972). Tetanic stimulation causes poorly sustained

tension, followed by little post-tetanic potentiation. Another preparation used for studying HC-3 and neuromuscular transmission is the rat isolated phrenic nerve-diaphragm, because of its relative insensitivity to postsynaptic effects (Bowman, Hemsworth and Rand, 1967). Recording intracellularly, Elmquist and Quastel (1965) demonstrated that at low concentrations, HC-3 caused a progressive decline in the size of miniature end plate potentials (m.e.p.p.s) and in the quantal components of the end plate potential as transmitter was released. When used as an isolated tissue preparation, the rat phrenic nerve diaphragm shows properties similar to other nerve-muscle preparations previously mentioned, except that choline is a poor antagonist in the presence of HC-3 (Rand and Chang, 1960).

Other peripheral sites used to study HC-3 have included autonomic neuroeffector junctions. At these sites HC-3 acts in a similar fashion as is seen at the somatic neuromuscular junction (Everett, 1968; Vanov, 1965; Rand and Ridehalgh, 1965; Appel and Vincenzi, 1970; Lindmar et al., 1980). In addition, inhibition of ACh synthesis by HC-3 has been demonstrated at sympathetic ganglia (Birks and McIntosh, 1961), and also at the sympathetically innervated guinea pig vas deferens (Chang and Rand, 1960).

The central actions of HC-3 after intraventricular (ivt) injection have also been studied. HC-3 produces a brief period of catatonia after ivt injection into conscious animals (Slater, 1968), followed occasionally by seizures (Shellenberger and Domino, 1967; Slater, 1968). Reduction of brain ACh levels by HC-3 has been correlated with an increased number of median trials to acquire a conditioned avoidance response in rats (Russel

and Macri, 1978). It has also been reported that HC-3 causes a behavioral response in rats similar to, but less severe than, that caused by septal lesions, the intraventricular dose being correlated to the intensity of the response (Freeman et al., 1979).

In addition to whole brain studies, the levels of ACh after HC-3 administration have been studied in areas of the brain believed to have a cholinergic component. HC-3 has been found to reduce ACh levels in the caudate nucleus (Hebb et al., 1964), cerebral hemispheres, midbrain, pons (Slater, 1969), cerebral slices (Richter, 1976), striatum, cortex, cerebellum and hippocampus (Freeman et al., 1979). In all of the above cases, the effects of HC-3 were antagonized by administration of choline chloride. HC-3 does not antagonize the active transport of choline through the blood-brain barrier, however, and it is presumed that this transport occurs via a different mechanism (Ross and Jenden, 1973).

Radioactive ^{14}C-HC-3 has also been used as a tool to locate sites of ACh synthesis in canine caudate nucleus and hippocampus (Sellinger et al., 1969), and in whole rat brain (Csillik, Haarstad and Knyihar, 1970), both utilizing autoradiographic techniques. In spontaneously hypertensive rats, intracerebroventricular injection of HC-3 has been shown to lower blood pressure (Brezenhoff and Caputi, 1980); and ivt administration of HC-3 produces a dose-dependent hypothermia in rats (Lin, Wang and Chandra, 1980).

HC-3 antagonizes the tremor produced by eserine in rats (Slater and Rodgers, 1968) and prevents the rise in brain ACh produced by this compound. Choline restores the tremors by reversing the inhibition of ACh

synthesis (Slater and Rodgers, 1968). In rats intoxicated with the organophosphorous anticholinesterase agent soman, intraventricular administration of HC-3 prevented a soman-induced rise in brain ACh levels, and protected against soman toxicity (Harris, Stitcher and Heyl, 1982).

In anaesthetized dogs, the fall in brain ACh caused by HC-3 has been associated with high voltage slow waves in the amygdala and neocortex, and inhibited EEG activation produced by electrical stimulation of the midbrain reticular formation, diffuse thalamic nuclei and posterior hypothalamus (Dren and Domino, 1968). The hippocampal immobile rythmic slow activity of the rat is severely attenuated by intraventricular injection of HC-3; this activity is restored by subsequent systemic administration of choline chloride (Robinson and Green, 1980).

In a study of the role of ACh in sleep, wakefulness and dreaming in cats, ivt injection of HC-3 facilitates slow wave sleep, but virtually abolishes paradoxical sleep (Hazra, 1970). HC-3 has also been investigated in the spinal cord. Iontophoretic application of HC-3 to Renshaw cells causes a dimunition in the number of spikes, and a delay of the first spike of the Renshaw cell discharge after ventral root stimulation (Quastel and Curtis, 1965; Craig, Curtis and Lodge, 1977). The change in the number of spikes is frequency dependent, but the increased latency is not, leading Craig et al. (1977) to propose both presynaptic and postsynaptic mechanisms of action in this preparation.

Several synthetic analogues of HC-3 have been studied mainly as an attempt to elucidate structure-activity relationships. Schueler (1955) reported that HC-15, which is exactly half the HC-3 molecule, exhibited no

toxic activity in mice in doses up to 50 mg/kg. Bowman, Hemsworth and Rand (1967) demonstrated that HC-15 possesses a postjunctional, nondepolarizing block on neuromuscular transmission in the rat phrenic nerve-diaphragm preparation, although it is much less active than HC-3. Hemsworth (1971) reported that HC-15 causes both a prejunctional and postjunctional block of transmission in the cat sciatic nerve-tibialis muscle preparation; and furthermore demonstrated an anticholinesterase action. In none of these actions is HC-15 as potent as HC-3.

Marshall and Long (1959) showed that the introduction of an ether or methylene linkage between the two phenyl rings of HC-3 produced only a minor decrease in potency. Subsequent studies have shown that the biphenyl moiety of HC-3 can be replaced by a hexamethylene chain (Powers, Kruger and Schueler, 1962), one phenyl ring (Thampi et al., 1966), or three phenyl rings (Gardiner and Sung, 1969) without appreciable loss of HC-3-like activity. Domer et al. (1980) synthesized a series of terphenyl compounds with HC-3-like activity, and found most of them to be as potent as their respective biphenyl analogues.

DiAugstine and Haarstad (1970) studied several methyl analogues of HC-3 and found hemicholinium-like activity, but less toxicity. Acetylation of HC-3 to produce the open ring acetyl secohemicholinium causes, in addition to the presynaptic blockade of choline transport, a more powerful direct inhibition of CAT, a systemic cholinomimetic activity, and a potentiation of catecholamine activity (Domino et al., 1973). Recently, 2-bromoethylamine (mustard) derivatives of both HC-3 and HC-15 have been synthesized. The HC-3 bromomustard effected greater inhibition of sodium

dependent high affinity choline uptake in rat brain synaptosomes than HC-3 (Smart, 1983), and is thus at present the most potent known synthetic inhibitor of this system. Synaptosomes treated to remove the drug showed maintained reduction in the Vmax of choline transport, and it has been suggested that HC-3 bromomustard forms a covalent bond with the transport mechanism, causing irreversible inhibition (Smart, 1983).

In summary, the mechanism of action, and pharmacological properties of HC-3 have been discussed. The principal action of HC-3 is the inhibition of high affinity choline transport leading to diminished ACh synthesis; in addition, HC-3 is also a weak anticholinesterase and a postsynaptic neuromuscular blocker.

Anticholinesterase Compounds

The enzyme acetylcholinesterase (AChE) is found at cholinergic terminals throughout the central and peripheral nervous systems where it hydrolyses ACh to choline and acetate, terminating the action of ACh. Anticholinesterase agents inhibit AChE causing ACh to persist at cholinergic synapses, leading to excessive stimulation of cholinergic receptors. There are two classes of anticholinesterases, "reversible" (carbamate type) and "irreversible" (organophosphates). Physostigmine (eserine), a carbamate anticholinesterase, is an alkaloid derived from the Calabar bean (the dried ripe seed of Physostigma venenosum Balfour), and early investigations of its pharamacological properties were conducted in mid-nineteenth century England (for historical review see Karczmar, 1970).

In the 1950s a series of heterocyclic, aromatic and napthyl carbamates were synthesized as insecticides and found to be potent anticholinesterases

with a high degree of selective toxicity (Gysin, 1954 as cited in Taylor(1980)). Carbaryl was the prototype of this latter group.

The first organophosphorous inhibitor synthesized was tetraethyl-pyrophosphate (TEPP), by DeClermont in 1854 (cited in Karczmar, 1970). Lange, in 1935, recognized the potential of organophosphorous agents as insecticides and fungicides, and prior to the Second World War, several compounds (e.g., parathion) were synthesized for this purpose by Schrader (cited in Karczmar, 1970). Subsequently, more toxic "war gases" such as tabun, soman and sarin were synthesized by Schrader's group under the direction of the German government (for review see Taylor, 1980). Investigators in Britain and America also searched for potentially toxic compounds, and diisopropylphosphorofluoridate (DFP) was synthesized by McCombie and Saunders (1946).

The active center of AChE contains a negative subsite called the anionic site and a catalytic "esteratic" site. The anionic site binds to the quaternary nitrogen while the serine hydroxyl group of the esteratic site breaks the ACh ester bond and is itself acetylated. Regeneration occurs via spontaneous hydrolysis to yield acetate (for detailed account see Usdin, 1970).

Physostigmine, neostigmine and other compounds with a methylcarbamate of a basically substituted simple phenol are hydrolysed by AChE in a reaction similar to that for ACh. The enzyme esteratic-site is, however, carbamylated (Wilson, Hatch and Ginsberg, 1960), and in contrast to the acetylated enzyme (which regenerates within a few thousandth's of a second) spontaneous regeneration occurs much more slowly. This is because the

carbamylated enzyme is far more stable and is thus unable to bind with ACh for the duration of carbamylation (Wilson and Harrison, 1961).

The organophosphates, like the carbamates, react at the esteratic site; the resultant phosphorylated enzyme being very stable. Compounds such as DFP are postulated to only react with the esteratic site, but compounds such as cholinylmethyl phosphono fluoridate are, in addition thought to react with the anionic site (Holmstedt, 1963; Usdin, 1970). Hydrolysis of the serine residue-phosphorous bond can occur at a very slow rate until a process called aging occurs. Aging occurs by dealkylation of the phosphorous atom to form a hydroxy group, thus stabilizing the enzyme-inhibitor complex further (Jansz, Brons and Waringa, 1959; Harris et al., 1966; Usdin, 1970).

Inhibitors of AChE have profound pharmacological effects in the peripheral and central nervous systems. In the periphery the effects are dependent on interference of cholinergic transmission at autonomic and somatic neuromuscular effector sites. In the autonomic system, vagal potentiation by tabun causes bradycardia (Holmstedt, 1951), although in dogs sarin and TEPP cause an increase in peripheral vascular resistance (DeBurgh and Wright, 1956), and in rats both sarin and DFP produce hypertension when given in sub-lethal doses (Dirnhuber and Cullumbine, 1955).

Duodenal activity is susceptible to anticholinesterase action; in dogs, both sarin and tabun increase tonus and rythmicity at doses producing no discernable effects on respiration or circulation (Krop and Kunkel, 1954). Increasing doses of DFP administered to the isolated rabbit

duodenum produces spastic contraction (Shelley, 1955), the spleen of the dog is contracted by TEPP (Scott, 1957), and chronic administration of organophosphates produces gastric ulcers in various experimental animals (Holmstedt, 1959). Other autonomic, parasympathetic actions of anticholinesterases include increased salivation (Emmelin and Stromblad, 1958), miosis (Sanderson, 1957), and bronchoconstriction (Krop and Kunkel, 1954).

At the neuromuscular junction, anticholinesterases produce twitch potentiation, fasiculations, tetanic inhibiton (Wedensky inhibition), and have an anti-curare action (Hobbiger, 1976). One preparation used extensively for studies of anticholinesterases on neuromuscular transmission has been the rat isolated phrenic nerve-diaphragm preparation (Hobbiger, 1976). Early studies showed that TEPP first enhances, then decreases single twitch response of the preparation (Burgen, Keele and Slome, 1949). Further studies showed that the response to TEPP, DFP and paraoxon was characterized by enhanced single twitches, fasiculations and tetanic fade (Barnes and Duff, 1953). Barnes and Duff (1953) went on to measure AChE activity at various stages of paraoxon poisoning. They calculated that twitch potentiation starts when AChE is 60% inhibited and the preparation returns to normal at 95% inhibition.

Van der Meer and Meeter (1956a) studied the effects of DFP on the rat phrenic nerve-diaphragm preparation. The contraction amplitude of the muscle, when stimulated indirectly at 0.5 Hz was initially increased by DFP, then returned to pre-DFP values. Cholinesterase activity of the muscle was found to be 20% of control when single twitch potentiation

began, and was completely inhibited when contractions returned to pre-DFP values. Preparations not given nerve stimulation showed spontaneous contraction at 80% cholinesterase inhibition (Van der Meer and Meeter, 1956a), these being postulated to occur via an antidromic reflex initiated by a presynaptic action of ACh (Randic and Straughan, 1964).

Antagonism of tetanic contractions (tetanic inhibition) of the rat phrenic nerve-diaphragm preparation by DFP has also been documented (Barstad, 1960). The preparations were stimulated for a period of 5 seconds at either 60 Hz or 120 Hz, and following addition of DFP the tetanic contractions of the muscle were abolished within 11 minutes. Cholinesterase activity determinations showed that activity of 10-15% of normal is sufficient to sustain 60 Hz stimuli, and 15-20% activity is sufficient to sustain 120 Hz stimuli (Barstad, 1960). Using the same preparation, Fleischer et al. (1960) demonstrated tetanic inhibition at 80 Hz and showed reversal of inhibition with TMB-4, an oxime reactivator. More recent work using paraoxon has shown that once tetanic fade starts to occur at a given percent reduction of cholinesterase activity, further small losses of AChE activity produce large reductions in tetanic response (Heffron and Hobbiger, 1979). In vivo nerve-muscle preparations have also been used to study anticholinesterase activity. Procedures include the cat anterior tibialis and cat sciatic-gastrocnemius preparations (for review see Werner and Kuperman, 1963); effects similar to that seen with the rat phrenic nerve diaphragm have been observed after close arterial injection (Hunt, 1947).

Anticholinesterases also affect cholinergic components of the CNS.

The CNS pharmacology has been extensively reviewed by Karczmar (1967) and is briefly summarized below. DFP causes electrocortical arousal in monkeys and cats and lowers the sensory and electrical threshold of the reticular formation. Evidence also exists that anticholinesterases depress thalamocortical recruitment, produce an arousal pattern in the hippocampus, and depress cochlear responses to sound. The discharge of Renshaw cells are prolonged by anticholinesterases and the respiratory paralysis produced by DFP has been shown to be predominantly caused by a central component (for review see Holmstedt, 1959 and Karczmar, 1967).

The toxicological symptoms of anticholinesterase poisoning are manifested by the effects of excess ACh at peripheral and central muscarinic and nicotinic receptor sites. Symptoms caused by excessive muscarinic stimulation include miosis, blurred vision, rhinorrhea, bronchoconstriction, abdominal cramps, diarrhea, sweating, involuntary micturition, increased salivation and lacrimation, and bradycardia (Holmstedt, 1959). The neuromuscular junction and autonomic ganglia are susceptible to excessive nicotinic stimulation, which causes fasciculations, muscular twitching, and mild elevation of blood pressure. In higher doses the fasciculations become widespread, and muscular weakness and eventual failure occur (Holmstedt, 1959). The central manifestations of toxicity have been reviewed by Wills (1970); briefly, they are anxiety, dizziness, confusion, ataxia, coma and generalized convulsions. The EEG resembles the spiking seen in grand mal epilepsy.

The drug of choice in treatment of anticholinesterase poisoning is atropine, which blocks muscarinic receptors. In addition, oxime

reactivators (e.g., pyridine-2-aldoxime or 2-PAM), which displace organophosphates from the esteratic site of the enzyme to restore activity, may prove useful therapy. Reactivators cannot, however, displace the organophosphate once aging has occurred (for review see Wills, 1970; Taylor, 1980), and 2-PAM does not cross the blood brain barrier (Ellin, 1982), thus limiting the therapeutic value of this oxime.

Other approaches to treatment of organophosphate poisoning have included the use of carbamates. Pyridostigmine has been shown to reverse soman induced tetanic inhibition of the rat phrenic nerve-diaphragm preparation (French, Wetherell and White, 1979), and physostigmine has been demonstrated to have protective value against soman toxicity in rats (Harris, Stitcher and Heyl, 1980). The mechanism of actions of carbamates is not fully understood although Green (1983) has proposed that kinetic factors alone can account for the protective action. Pretreatment with several other compounds has been shown to increase the LD_{50} of organophosphates. Such compounds include O-3-(trimethylammonio)-phenyl-1,3,2-dioxaphosphorinane-2-oxide iodide (TDPI) (Ashani et al., 1983), soman-simulator (Wolthius, Benschop and Berends, 1981), and sodium phenobarbital (Clement, 1983). Unfortunately none of these compounds are of therapeutic value when administered subsequent to organophosphate exposure.

Recognizing the potential of inhibitors of ACh synthesis as organophosphate antagonists, Harris, Stitcher and Heyl (1982) gave intraventricular injections of HC-3 to rabbits, prior to intoxication with soman, and found that HC-3 increased the LD_{50} of soman several-fold. HC-3

has also been shown to antagonize the tetanic inhibiton of the rat isolated phrenic nerve-diaphragm preparation following DFP *in vitro* or soman *in vivo* (Grieve and VanMeter, 1983).

Investigations into possible antagonists of anticholinesterase poisoning, are hampered by the fact that anticholinesterases produce effects unrelated to inhibiton of the AChE enzyme. Organophosphates cause a variety of metabolic changes (for review see Ellin, 1982), but most complications arise from direct effects on post synaptic receptors. Studies on neuromuscular transmission have shown that in the presence of DFP inhibited AChE, end plate current amplitude was restored despite continued 100% enzyme inhibition (Kuba and Albuquerque, 1973). DFP administered to rabbits produces EEG seizures in the presence of previously inhibited AChE (VanMeter, Karczmar and Fiscus, 1978). Both groups proposed that these observations were due to a direct action of DFP. Using patch clamp techniques, Shaw, Akaike and Albuquerque (1983) have demonstrated a direct action of both pyridostigmine and physostigmine on the acetylcholine receptor-ionic channel complex.

In addition to the prompt effects noted above, some organophosphates also produce delayed neurotoxicity. This effect is characterized by degeneration and demyelination of long fibers, and is postulated to occur because of inhibition of neurotoxic esterase (Johnson, 1974). Clinical symptoms include weakening of distal muscles in limbs 6-14 days after exposure to organophosphates (for review of delayed neurotoxicity, see Abou-Donia, 1981).

The biochemical, pharmacological and toxicological effects of

anticholinesterase agents described above are of interest because of the need for effective therapy following organophosphate poisoning. The rat isolated phrenic nerve-diaphragm preparation is a useful model of neuromuscular transmission, enabling study on the effects of organophosphates, and their potential antagonists.

METHODS AND MATERIALS

Synthesis of a Tertiary Hemicholinium

As tertiary hemicholiniums are not commercially available, one such compound was obtained by organic synthesis. The procedure used, which was based on the method of Long and Schueler (1954) as modified by DiAugustine and Haarstad (1970), involved a three-stage synthesis beginning with a Friedel-Crafts addition of bromoacetylbromide onto biphenyl. Biphenyl (25 g ,0.16 Moles) was dissolved in 150 ml of carbon disulfide in a 500 ml, three necked, round bottomed flask. The solution was cooled and 60 g of anhydrous aluminum chloride was slowly added while shaking. The mixture was then refluxed while slow addition of 80 g (0.4 Moles) bromoacetylbromide caused liberation of HBr. After 2 hours, the reaction mixture was poured into a mixture of cracked ice, methanol and hydrochloric acid and stirred. The white product was collected on a filter paper and dried. A pure reaction product was obtained after two recrystallizations from tetrahydrofuran (THF) in 28% yield.

To a solution of 6.0 g (0.015 Moles) of the product (4,4'-dibromoacetyl biphenyl) dissolved in 30 ml of boiling THF, 3.0 g of (0.03 Moles, 3.6 ml) of 4-methylpiperidine was added slowly. The mixture was cooled, allowed to stand for 30 min, and the crystals which formed were collected on a filter paper, dissolved in absolute ethanol and precipitated through addition of anhydrous ether (Yield, 14-18%).

The product of this reaction (1.0 g) was then dissolved in water, and an excess (0.75 g) of sodium borohydride added. The free base of the tertiary hemicholinium crystallized out of solution, was filtered, dried

and recrystallized from THF, dried again and stored for use (Yield, 46%). Structures of all products were confirmed by nuclear magnetic resonance spectroscopy (^{1}H nmr($CDCl_3$, TMS as internal standard) 0.8-1.7 m, 4-methylpiperidine, 2.4 d, CH_2, 4.7 t, CH, 7.5 d, biphenyl; ms 436.32 (calcd. 436.19)).

Dissection

To eliminate error due to differences between sexes and to ensure stable preparations, only young female rats, weighing between 150-250 g were used. Diaphragms from rats larger than 250 g were frequently found to be unstable. The animals (Sprague-Dawley or Sprague-Dawley and Norwegian crossbred rats) were killed by cerebral concussion followed by exsanguination. The left thoracic wall was completely removed, the phrenic nerve was ligated and cut cranial to the ligature. The diaphragm was attached to a tissue holder with cotton thread, and another long section of cotton thread was tied to the central tendon. A fan-shaped slice of diaphragm with the phrenic nerve attached was removed from the animal, and placed in a 50 ml isolated tissue bath filled with Krebs' solution (see Appendix A) at 37^{o}C and aerated with 95% O_2/5% CO_2 (see Fig. 1). The thread tied to the central tendon was attached to a Gould 33-03-981 isotonic transducer coupled to a Gould Brush 220 recorder. The phrenic nerve was placed in contact with platinum wire electrodes, and given cathodal stimulation, at a frequency of 1 Hz, with supramaximal voltage pulses of 1 msec duration delivered by a Grass SD9 stimulator. Tissues were allowed to equilibrate in the bathing solution for 20-30 min to ensure stable responses to nerve stimulation.

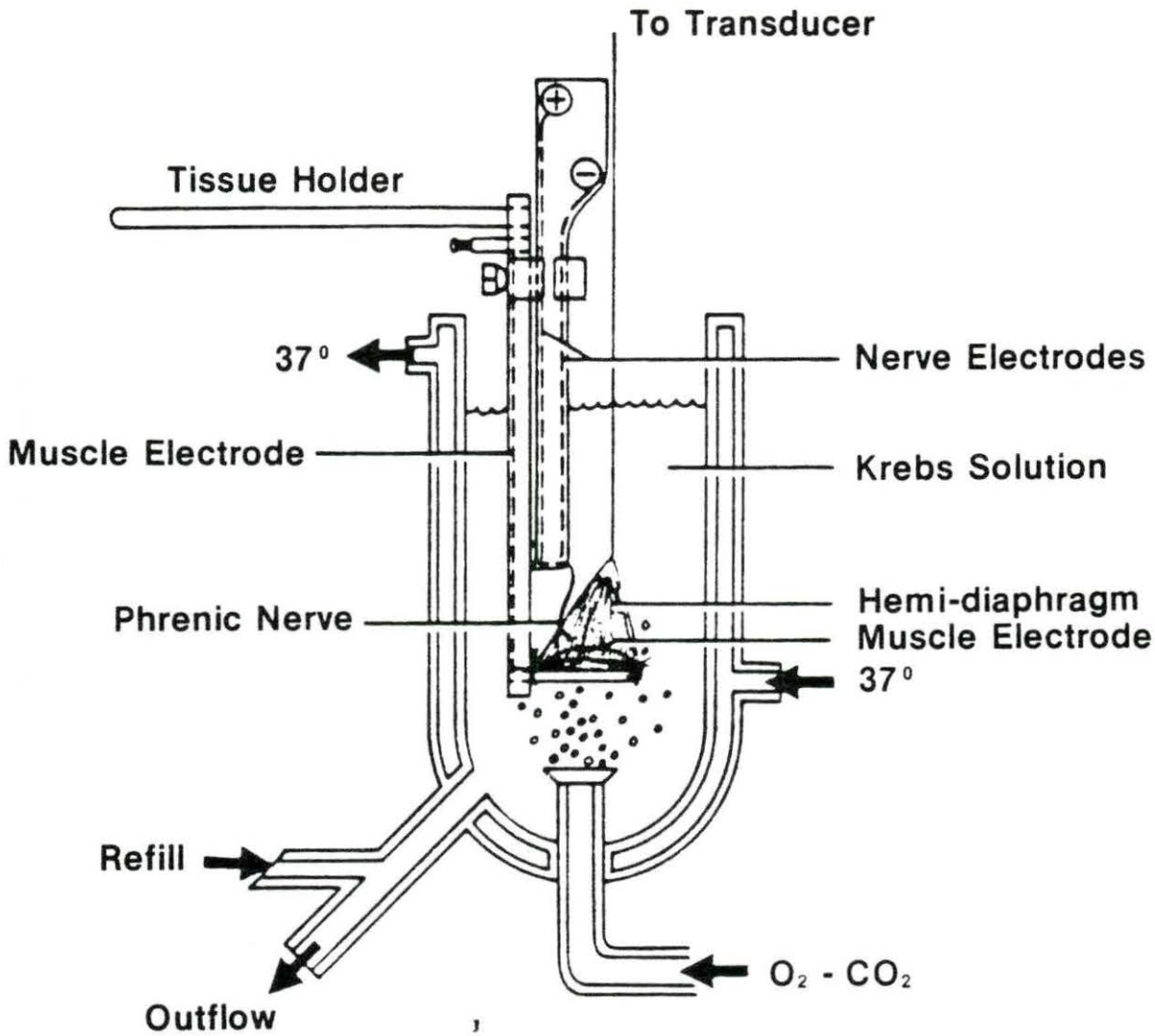


Fig.1. The rat phrenic nerve-diaphragm preparation

Data Recording and Analysis

Data were recorded with an ink-writing, rectilinear Gould Brush 220 recorder onto Gould Accuchart paper at a speed of 25 mm per minute. The paper was divided into primary squares of 0.5 cm and secondary squares of 1.0 mm. In studies evaluating antagonism of the response of the preparation to HC-3, and antagonism of the response to HC-3 by choline, the parameter measured was the time required to reach 50% of control single twitch contraction amplitude (T_{50}). As the response to 16.0 µg/ml HC-3 was assumed to be maximal, in each preparation, the T_{50} obtained with 16.0 µg/ml HC-3 was normalized to 1.00. Thus in each preparation, the T_{50}'s obtained at all HC-3 doses were divided by the T_{50} obtained with 16.0 µg/ml HC-3 to yield I_{50} values. The mean and standard error of I_{50} values (at each dose of HC-3) from different preparations were then computed.

Antagonism Studies

The known ability of HC-3 to deplete presynaptic cells of ACh makes this compound a potential physiological antagonist to organophosphates, which inhibit AChE and thus cause accumulation of ACh. In order to investigate depression of the preparation by organophosphates and physiological antagonism of this depression by HC-3, the following parameters were used: 1) amplitude of pretetanic single twitches, 2) amplitude of 50 Hz tetanic contractions of 5 second duration, and 3) amplitude of post-tetanic single twitches as parameters. In DFP studies, 50 Hz tetanic contraction amplitude was measured against control values to yield a % control value. In soman studies, the amplitude of contraction in

response to tetanic stimulation (T) was divided by the pretetanic single twitch amplitude to yield a T/S ratio, which is a measure of tetanic blockade. In both DFP and soman studies, the post-tetanic single twitch amplitude (P) was divided by pre-tetanic single twitch amplitude (S) to give a P/S ratio. The P/S ratio is a measure of post-tetanic potentiation or post-tetanic depression.

Drug Applications--In Vivo

Young adult female rats weighing 150-250 g were injected subcutaneously with 80, 160, or 320 μg/kg soman prior to experiments with the isolated phrenic nerve-diaphragm preparations, and the left phrenic nerve-diaphragms were removed as described previously 30 minutes post injection or on death.

In experiments determining the LD_{50}'s of HC-3 or the tertiary hemicholinium (tHC-3), rats were injected subcutaneously on the dorsal side of the abdomen. Animals which had not expired within 24 hours were assummed to be survivors.

Drug Applications--In Vitro

Drugs were added directly to the bathing solution (for protocols, see Appendix B) and were removed by complete evacuation of the tissue bath, and replacement with fresh Kreb's solution.

RESULTS

HC-3 was administered in increasing doses until a 50% reduction in contraction amplitude (from 1.0 Hz stimulation) was observed. The mean dose at which this occurred, the minimum effective dose, was determined to be 30 μg/ml (n = 15 preparations).

In order to determine if the observed reduction in contraction amplitude was due to a presynaptic mechanism, HC-3 (40 μg/ml) was administered to the preparation during nerve stimulation frequencies of 1.0 Hz and 0.2 Hz. HC-3 caused a progressive blockade of the preparation at 1.0 Hz, but this effect was not seen at 0.2 Hz (Fig. 2).

HC-3 did not cause blockade of the rat diaphragm in a dose-dependent manner when initially applied. Repeated doses of 40 μg/ml HC-3 (followed after each dose by washing out of drug and full restoration of initial contraction amplitude) caused the time required to reduce contraction amplitude 50% (T_{50}) to become successively shorter with each dose. Eventually, an equilibrium was reached where the maximum effect of HC-3 was seen at approximately a 16 μg/ml dose (i.e., doses higher than 16 μg/ml HC-3 did not further reduce T_{50}). Doses of HC-3 lower than 16 μg/ml produced responses in a dose-dependent manner (Fig. 3).

Choline chloride (8.0 μg/ml) applied to the preparation in the presence of HC-3 (4.0, 6.0, 8.0 μg/ml) at T_{50} antagonized the decrease in contraction amplitude. The effects of HC-3 at doses higher than 8.0 μg/ml were not antagonized by choline chloride unless HC-3 was first washed out. Antagonism of HC-3 by choline was also demonstrated when choline was administered prior to HC-3 (Fig. 4). Pre-administration of choline

Fig. 2. Effect of HC-3 (40 μg/ml) on single twitch contractions of rat diaphragm response to 1.0 Hz (Trace I, II) and 0.2 Hz (Trace III) stimulation of the phrenic nerve

I. HC-3 (40 μg/ml) causes a progressive reduction in amplitude of diaphragm contraction in response to 1.0 Hz stimulation; following wash-out of drug (w), contraction amplitude partially recovers.

II. Recovery of contraction amplitude occurs after administration of choline chloride (Ch, 8.0 μg/ml); this effect persists after wash-out of choline chloride (w).

III. At a stimulation frequency of 0.2 Hz, HC-3 (40 μg/ml) fails to induce reduction in contraction amplitude.

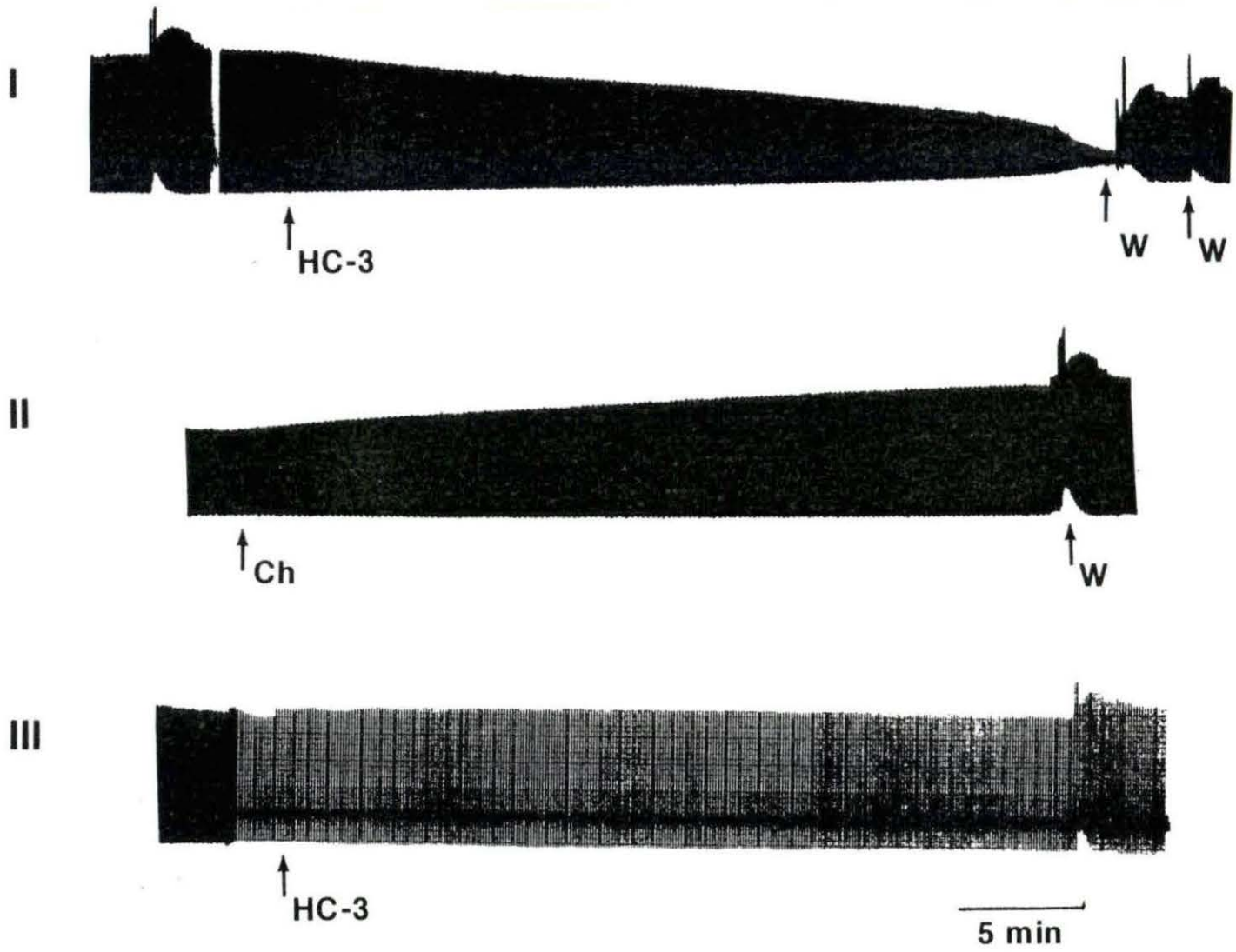

I
HC-3
W
W
II
Ch
W
III
HC-3
5 min

Fig. 3. Relationship between HC-3 and I_{50} is dose-dependent

Using a stimulation frequency of 1.0 Hz, HC-3 reduces I_{50} in a dose-dependent manner. The graph shows the mean $\pm$ s.e.m.

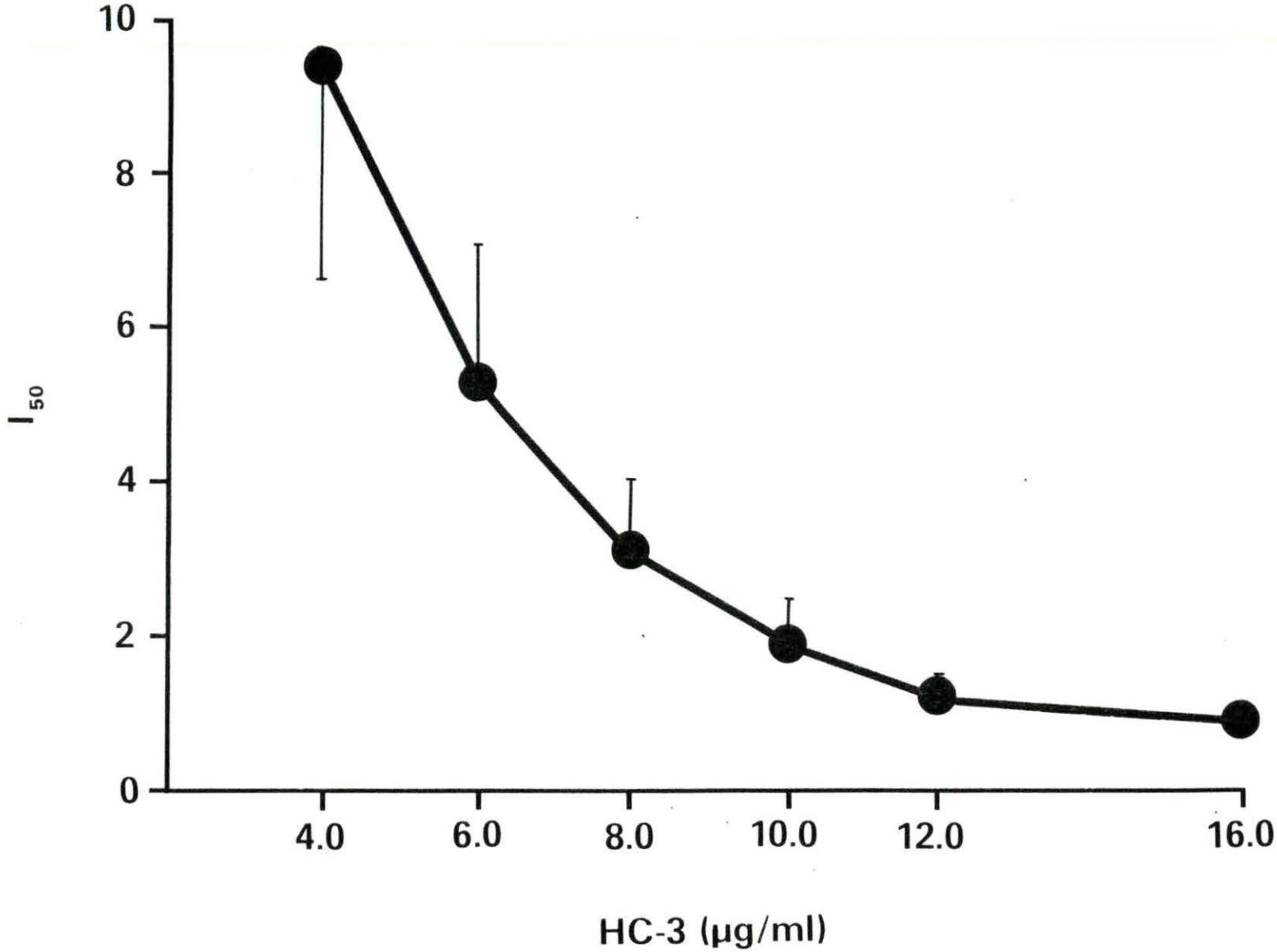
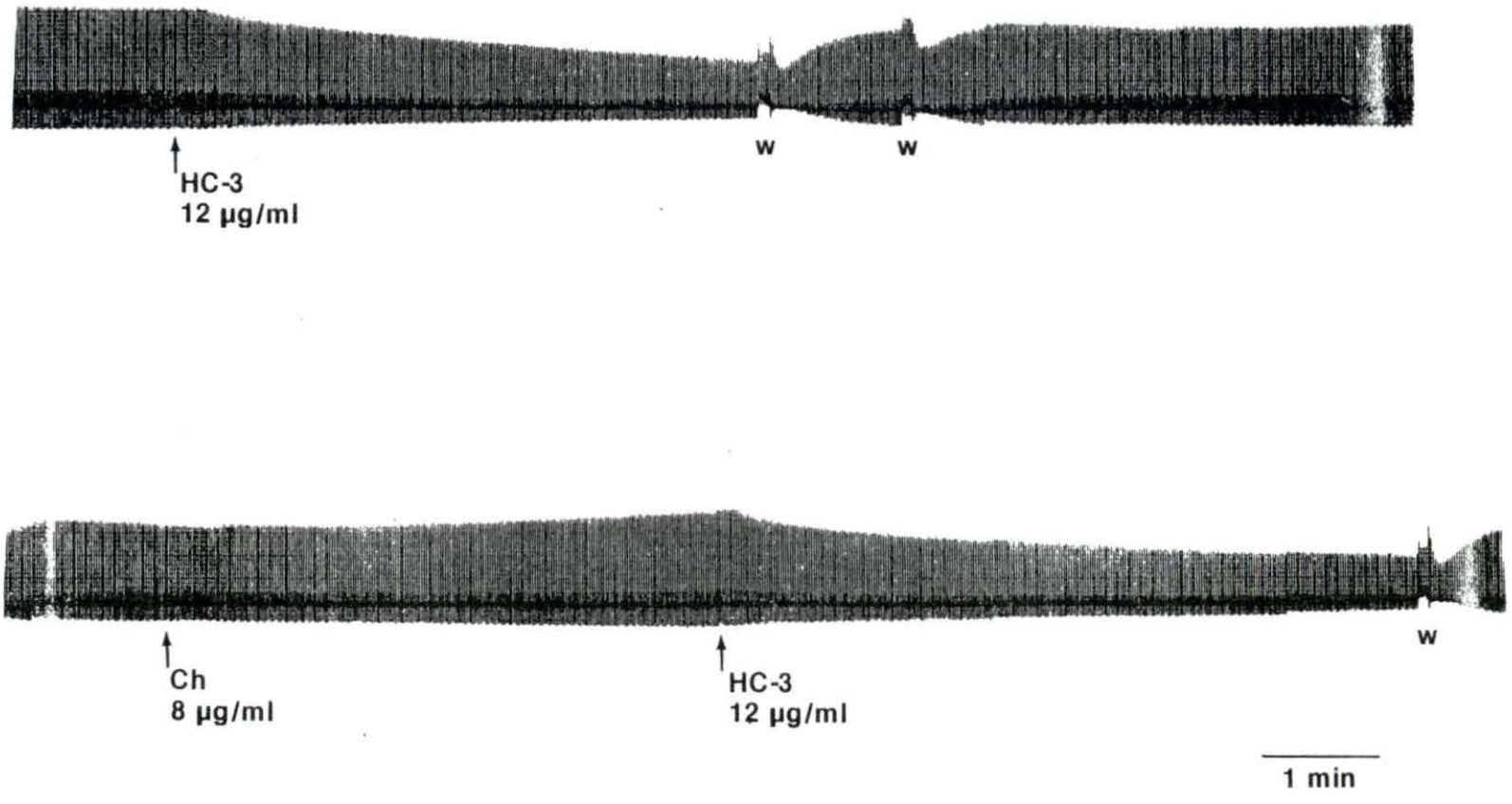

10
8
6
4
2
0
I_{50}
4.0
6.0
8.0
10.0
12.0
16.0
HC-3 (µg/ml)

Fig. 4. Choline chloride (8.0 μg/ml) antagonizes HC-3 (12.0 μg/ml) depression of diaphragm contraction amplitude

Upper trace: HC-3 (12.0 μg/ml) causes a 50% reduction of contraction amplitude after 3.7 min contact (T_{50} = 3.7 min)

Lower trace: Choline chloride (Ch, 8.0 μg/ml) induces recovery of contraction amplitude, still depressed after HC-3, and antagonizes the effects of HC-3 (12.0 μg/ml) by increasing T_{50} from 3.7 min to 4.8 min


HC-3
12 µg/ml
W
W
Ch
8 µg/ml
HC-3
12 µg/ml
W
1 min

chloride (8.0 μg/ml) increased the I_{50} compared to controls (Fig. 5), but was only effective when the dose of HC-3 was 4.0-8.0 μg/ml.

DFP Studies

Applications of 50 Hz stimulation to the phrenic nerve for 5 seconds produced tetanic contractions 2-3 times the amplitude of the preceding single twitch. Immediately after cessation of 50 Hz stimulation, a post-tetanic potentiation of 1.5-2 times the amplitude of pretetanic single twitches was also seen. DFP was applied first at a dose of 2.0 μg/ml as a sensitivity test, and subsequently at a dose of 1.0 μg/ml in conjunction with an HC-3 or saline challenge.

DFP (2.0 μg/ml) caused a reduction in post-tetanic potentiation after 7 min contact, and both a depression of tetanic contraction amplitude and post-tetanic depression were seen after 14 min exposure (Fig. 6). Preparations recovered 30 min after DFP was washed out (Fig. 6). Subsequent administration of 1.0 μg/ml DFP, followed 1 min later by saline caused greater tetanic depression, and post-tetanic depression than did 2.0 μg/ml DFP. However, if 1.0 μg/ml DFP was followed 1 min later by HC-3 (40.0 μg/ml), tetanic depression was reduced, and post-tetanic depression was absent (Fig. 7). After DFP (1.0 μg/ml) was washed out, amplitude of tetanic contractions in preparations given HC-3 recovered within 15 min, whereas recovery took 30 min in preparation given saline (Fig. 7).

Results from nine experiments demonstrated that HC-3 (40 μg/ml) gave significant protection against DFP depression of 50 Hz tetanic contraction amplitude (Fig. 8), and also protected against DFP depression of post-tetanic potentiation (Fig. 9).

Fig. 5. Effect of choline chloride on the dose-dependent relationship between HC-3 and I_{50}

HC-3 causes a dose-dependent decrease in I_{50} (shaded bars). Choline chloride (8.0 μg/ml) antagonized the effects of HC-3 (open bars) by significantly increasing the I_{50} over control values; graph shows mean ± s.d. ($^{*}p < 0.05$).

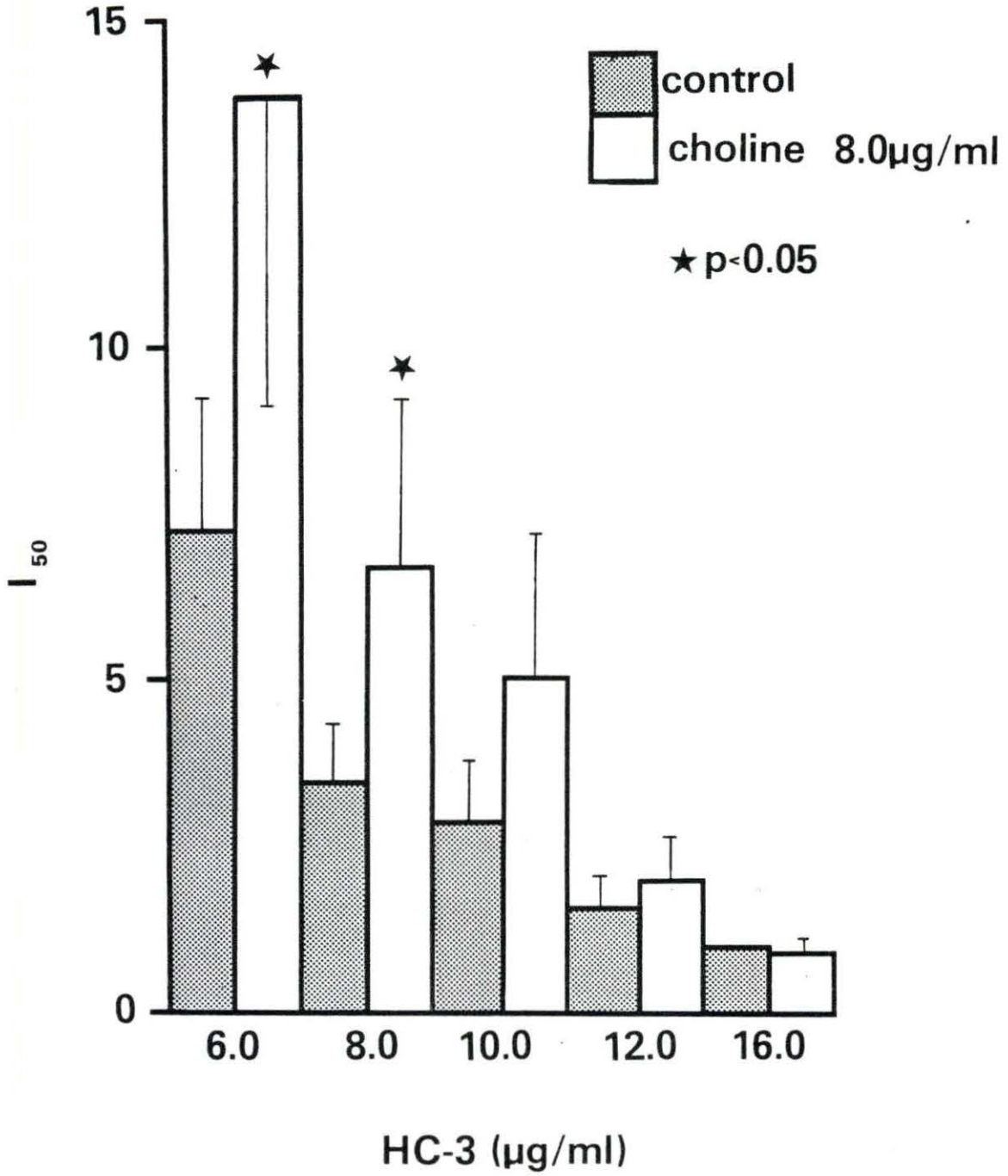

15
10
5
0
I_{50}
control
choline 8.0µg/ml
★ p<0.05
6.0
8.0
10.0
12.0
16.0
HC-3 (µg/ml)

Fig. 6. DFP (2.0 μg/ml) depresses 50 Hz tetanic contraction amplitude and post-tetanic potentiation

Note depressed tetanic and post-tetanic contraction amplitudes after 14 min contact time (arrow) compared to controls (CT). Preparations A and B both recover after DFP wash-out (lower traces A and B).

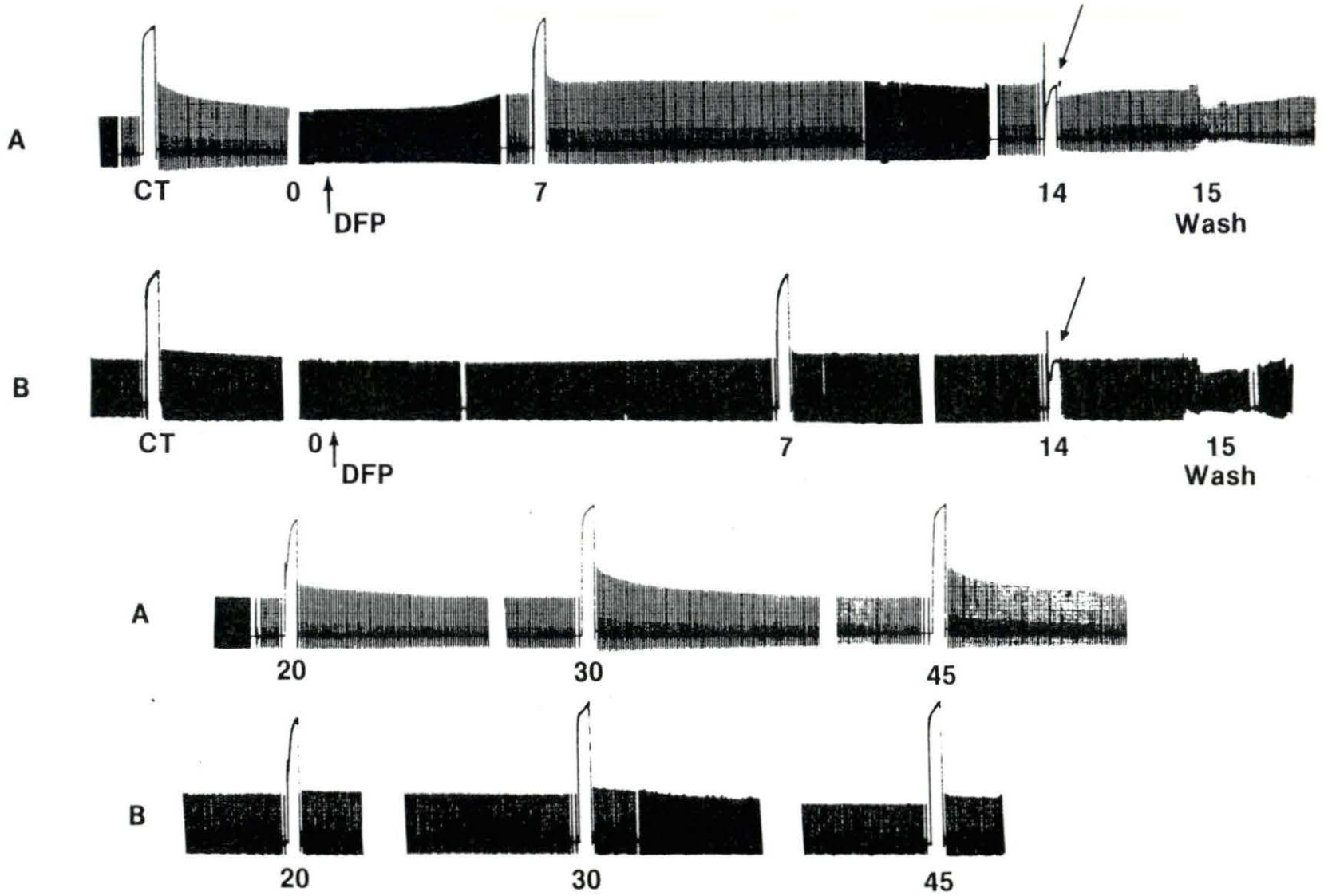

A
CT
0
DFP
7
14
15
Wash
B
CT
0
DFP
7
14
15
Wash
A
20
30
45
B
20
30
45

Fig. 7. DFP (1.0 μg/ml) depression of 50 Hz contraction amplitude and post-tetanic potentiation is antagonized by HC-3

Note difference in tetanic amplitude (arrows, a) and post-tetanic contraction amplitude (arrows, b) between preparations A and B. Recovery after DFP wash-out takes longer in preparation B (given saline,SAL).

N.B. This figure is a continuation of Fig. 6. Preparations are the same as shown in Fig. 6.

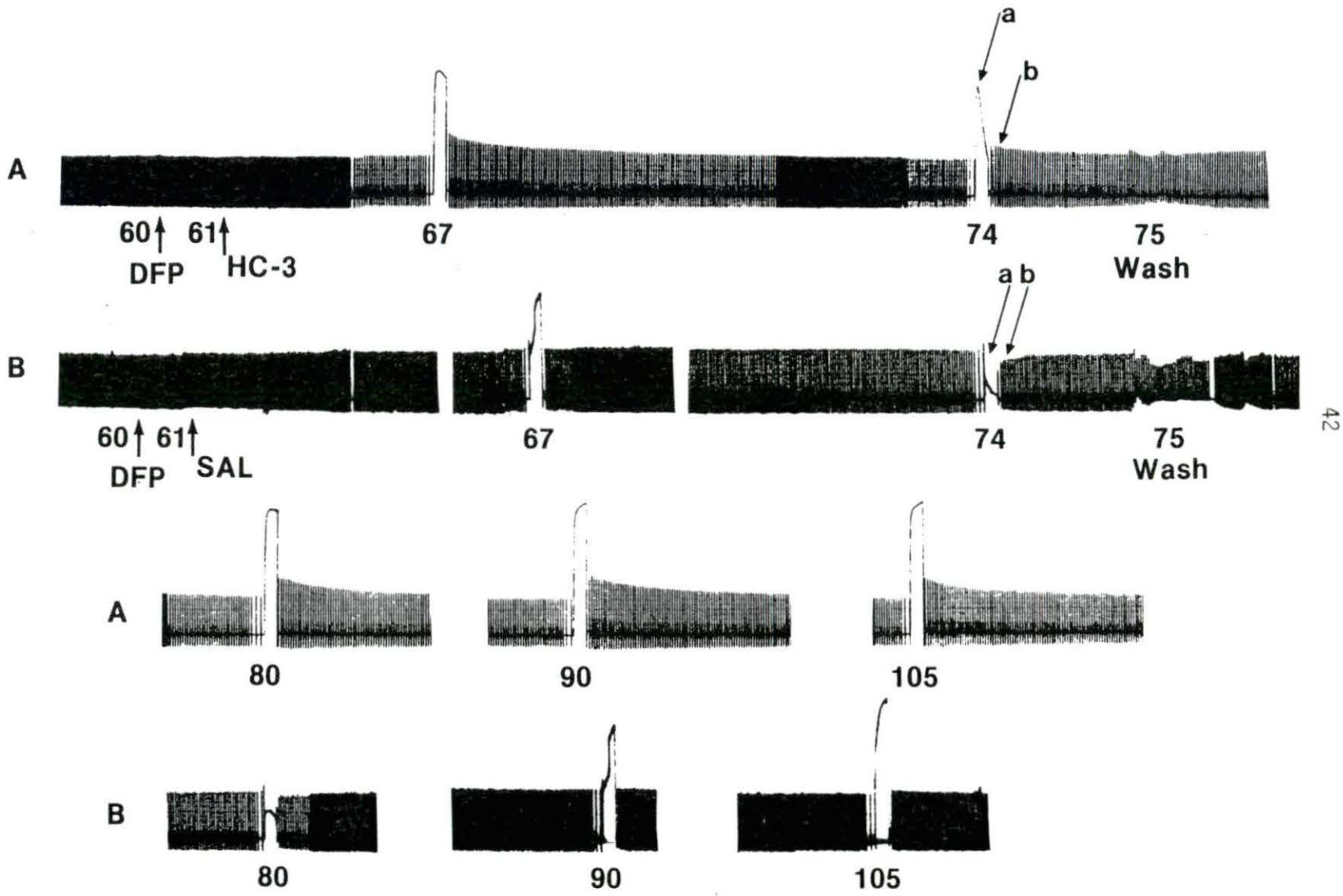

A
a
b
60
61
DFP
HC-3
67
74
75
Wash
B
a b
60
61
DFP
SAL
67
74
75
Wash
A
80
90
105
B
80
90
105

Fig. 8. DFP induced depression of tetanic contraction amplitude is antagonized by HC-3

After 14 min exposure, 2.0 μg/ml DFP (A) causes depression of 50 Hz tetanic contraction amplitude. After DFP wash-out, preparations recover. Subsequent exposure to 1.0 μg/ml DFP (B) followed by 40.0 μg/ml HC-3 causes significantly less depression of 50 Hz tetanic contraction amplitude than when DFP (B) is followed by saline.

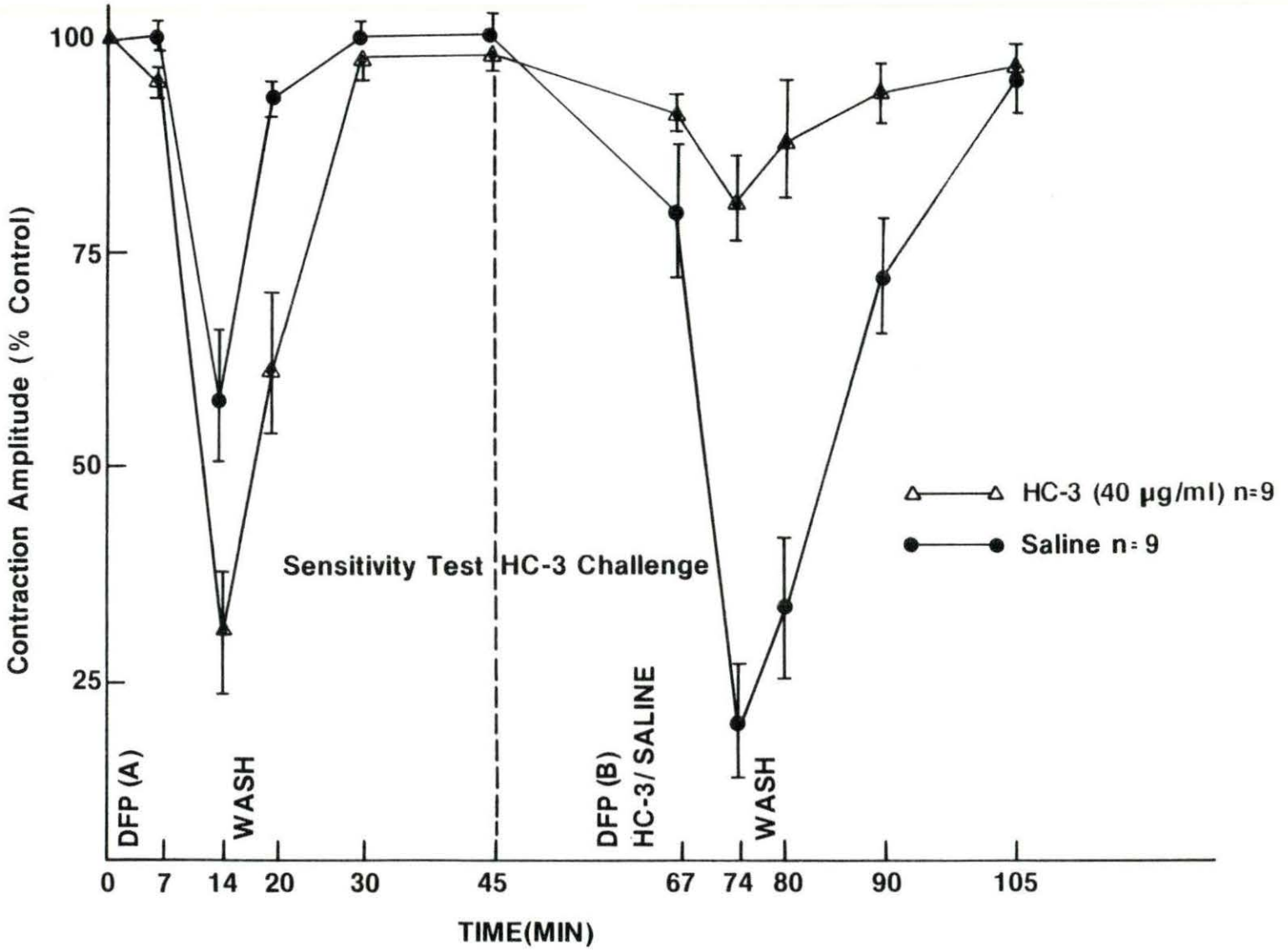
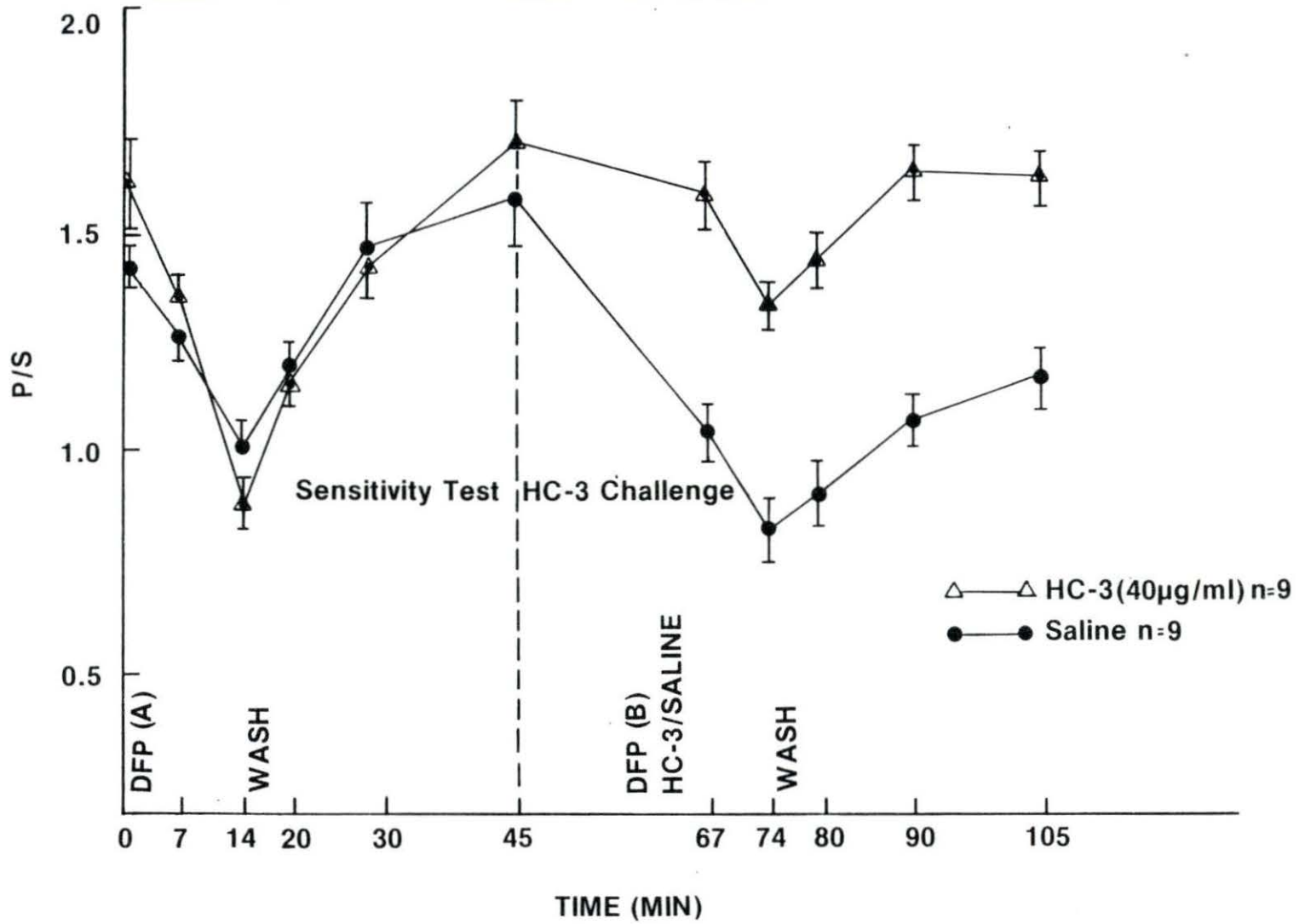

100
75
50
25
Contraction Amplitude (% Control)
DFP (A)
WASH
Sensitivity Test
HC-3 Challenge
DFP (B)
HC-3/ SALINE
WASH
0
7
14
20
30
45
67
74
80
90
105
TIME(MIN)
HC-3 (40 µg/ml) n=9
Saline n= 9

Fig. 9. DFP induced depression of P/S ratio is antagonized by HC-3

Administration of 2.0 μg/ml DFP (A) causes post-tetanic depression after 14 min exposure. After DFP wash-out, preparations recover. Preparations subsequently exposed to 1.0 μg/ml DFP followed by 40 μg/ml HC-3 show significantly less depression of post-tetanic contraction amplitude than preparations given 1.0 μg/ml DFP (B) followed by saline. The graph shows the mean ± s.e.m.


2.0
1.5
1.0
0.5
P/S
Sensitivity Test
HC-3 Challenge
DFP (A)
WASH
DFP (B)
HC-3/SALINE
WASH
HC-3(40µg/ml) n=9
Saline n=9
0
7
14
20
30
45
67
74
80
90
105
TIME (MIN)

Soman Studies

Preparations taken from animals administered with soman were examined. Application of a stimulation frequency of 50 Hz for 5 seconds to the phrenic nerve showed that soman (160 μg/kg and 320 μg/kg) caused a reduction in tetanic contraction amplitude as compared with preparations taken from animals given saline (Figs. 10, 11). Although administration of HC-3 (40 μg/ml) further reduced tetanic contraction amplitude after 13 min contact, 5 min after HC-3 was washed out tetanic amplitude increased over pre-HC-3 amplitude and continued to increase over a 25 min period (Figs. 10, 11). HC-3 caused post-tetanic depression during contact with the tissue, and, after HC-3 wash-out, post-tetanic contractions returned to pre-HC-3 amplitude. Administration of saline did not affect the preparations taken from rats receiving 160 μg/kg soman (Fig. 10); but preparations from rats receiving 320 μg/kg soman recovered slowly in the presence of saline (Fig. 11).

Soman (80 μg/kg) failed to significantly alter the response of preparations to 50 Hz stimulation as compared to preparations from rats receiving saline injections. Neither T/S ratio (Fig. 12) nor P/S ratio (Fig. 13) significantly differed from controls, nor did HC-3 (40.0 μg/ml).

The T/S and P/S ratios of preparations taken from rats receiving 160 μg/kg soman, on the other hand, were depressed as compared to saline injected controls. Exposure to HC-3 (40.0 μg/ml) for 6 min increased the T/S ratio (Fig. 14) but did not affect the P/S ratio (Fig. 15). After 13 min contact, HC-3 caused a depression of T/S ratio (Fig. 14) and P/S ratio (Fig. 15) compared with 6 min exposure. Once HC-3 was washed out, both the

Fig. 10. HC-3 induces recovery of the tetanic contraction amplitude of isolated diaphragms taken from rats given soman (160 μg/kg s.c.)

HC-3 (40.0 μg/ml) is administered to preparation A, and washed out 14 min later (A, upper trace). Note immediate recovery of tetanic contraction amplitude of preparation A (arrow) after exposure to HC-3; recovery is sustained after 25 min (A, lower trace). Saline does not significantly affect preparation (B, upper and lower traces).

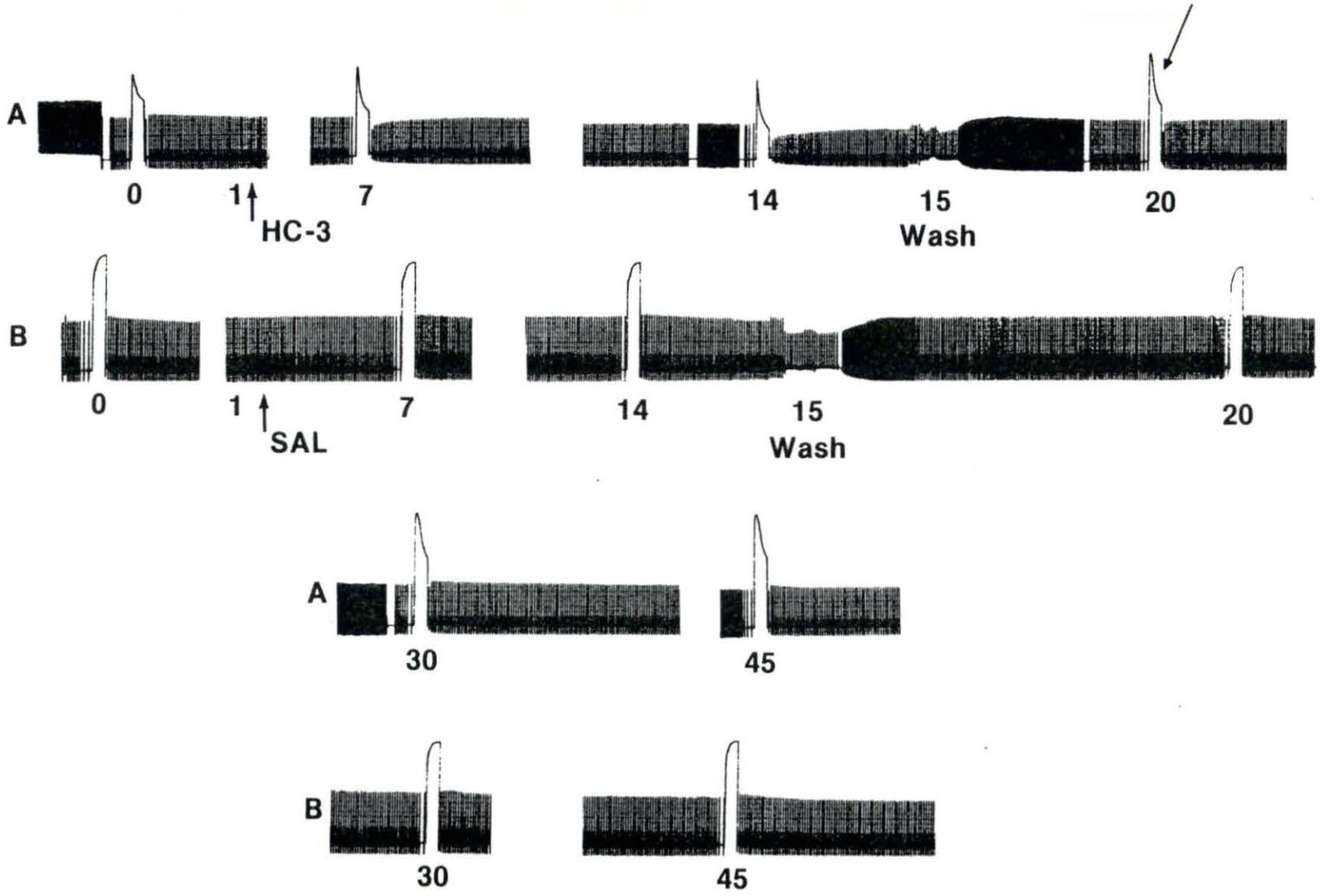

A
0
1
HC-3
7
14
15
Wash
20
B
0
1
SAL
7
14
15
Wash
20
A
30
45
B
30
45

Fig. 11. HC-3 increases rate of recovery of tetanic contraction amplitude in isolated diaphragms from rats given soman (320 μg/kg s.c.)

Preparation A is exposed to HC-3 (40.0 μg/ml) for 14 min (A, upper trace). Note immediate recovery of tetanic contraction amplitude after HC-3 wash-out (arrow, a), and recovery of post-tetanic potentiation (arrow, b). Recovery occurs slowly with saline (B, upper and lower traces) over a period of 45 min.

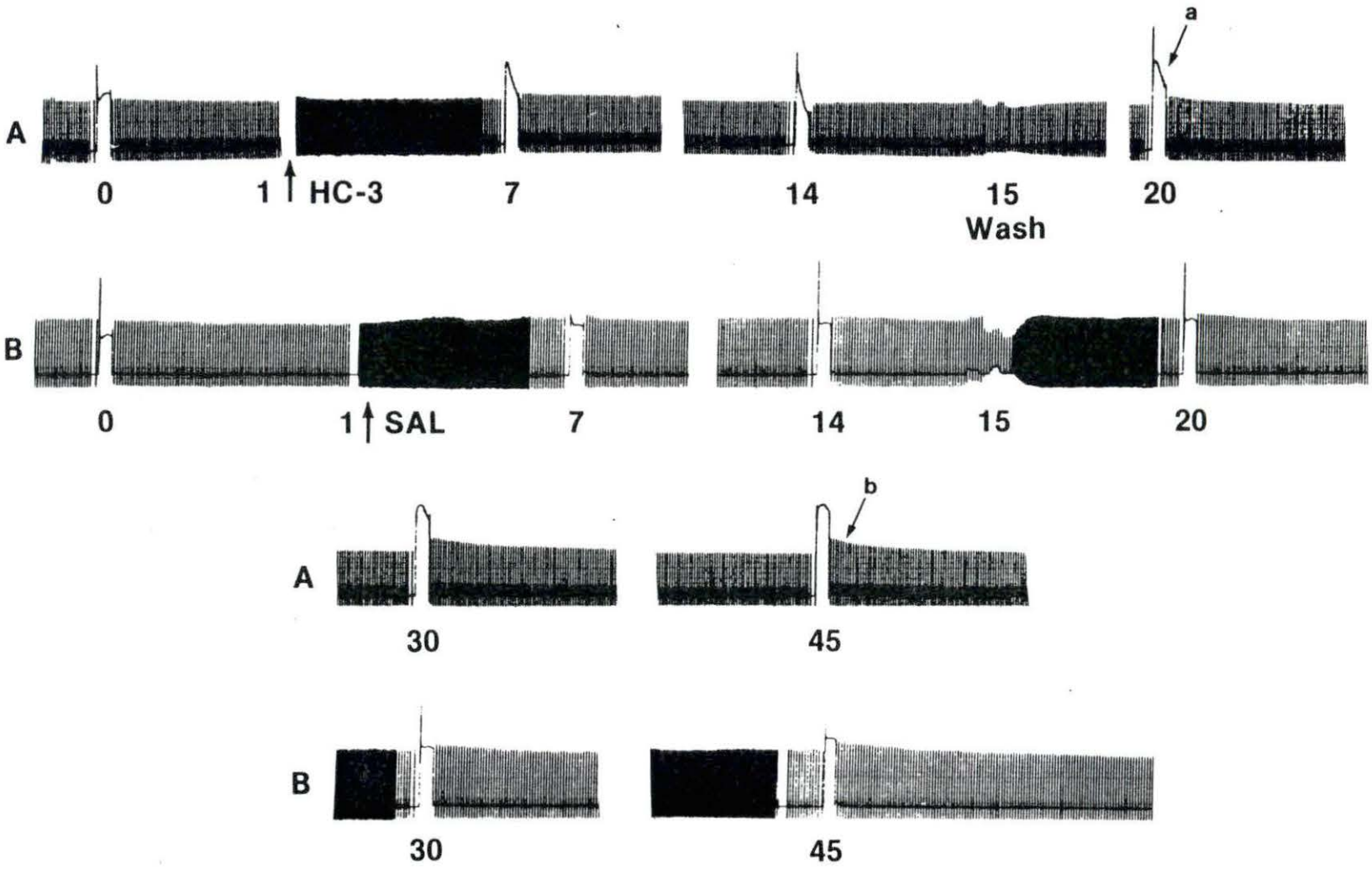

a
A
0
1
HC-3
7
14
15
Wash
20
B
0
1
SAL
7
14
15
20
b
A
30
45
B
30
45

Fig. 12. Soman (80 μg/kg s.c.) fails to alter T/S ratio of rat diaphragms placed *in vitro*

T/S ratio of diaphragms from soman (80 μg/kg) treated rats and control rats does not significantly differ. HC-3 (40 μg/ml) does not affect the T/S ratio of either group. The graph shows the mean ± s.e.m. ▲ - non-soman pretreatment (control). ■, ● - soman (80 μg/kg) pretreatment.

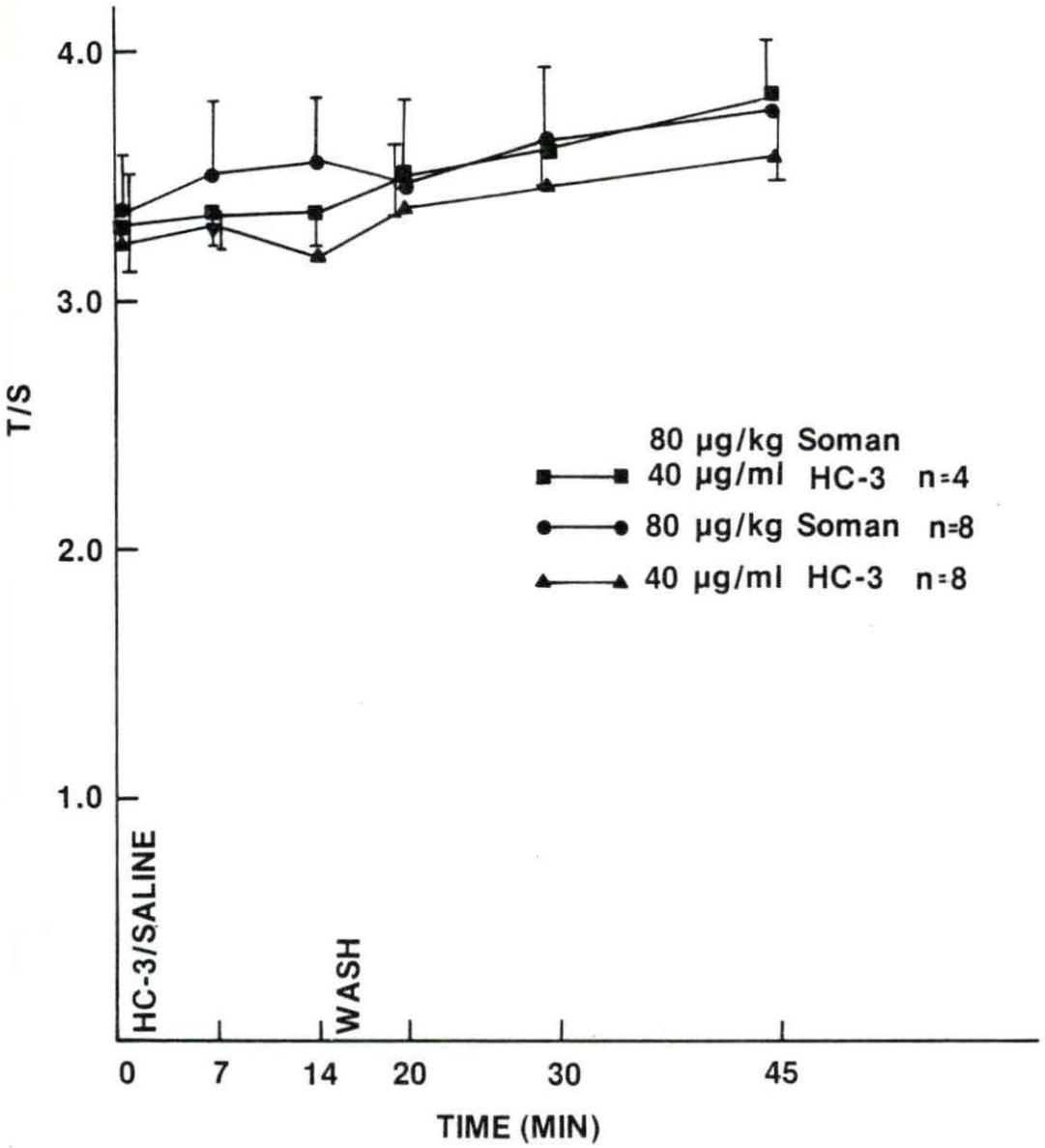

4.0
3.0
2.0
1.0
T/S
80 µg/kg Soman
40 µg/ml HC-3 n=4
80 µg/kg Soman n=8
40 µg/ml HC-3 n=8
HC-3/SALINE
WASH
0
7
14
20
30
45
TIME (MIN)

Fig. 13. Soman (80 μg/kg) fails to alter P/S ratio of rat diaphragms placed *in vitro*

P/S ratio of diaphragms from soman (80 μg/kg) treated rats and control rats does not significantly differ. HC-3 (40 μg/ml) does not significantly affect the P/S ratio in control or soman groups. The graph shows the mean ± s.e.m. ⬢ - non-soman pretreatment (control). ● , ■ - soman (80 μg/kg) pretreatment.

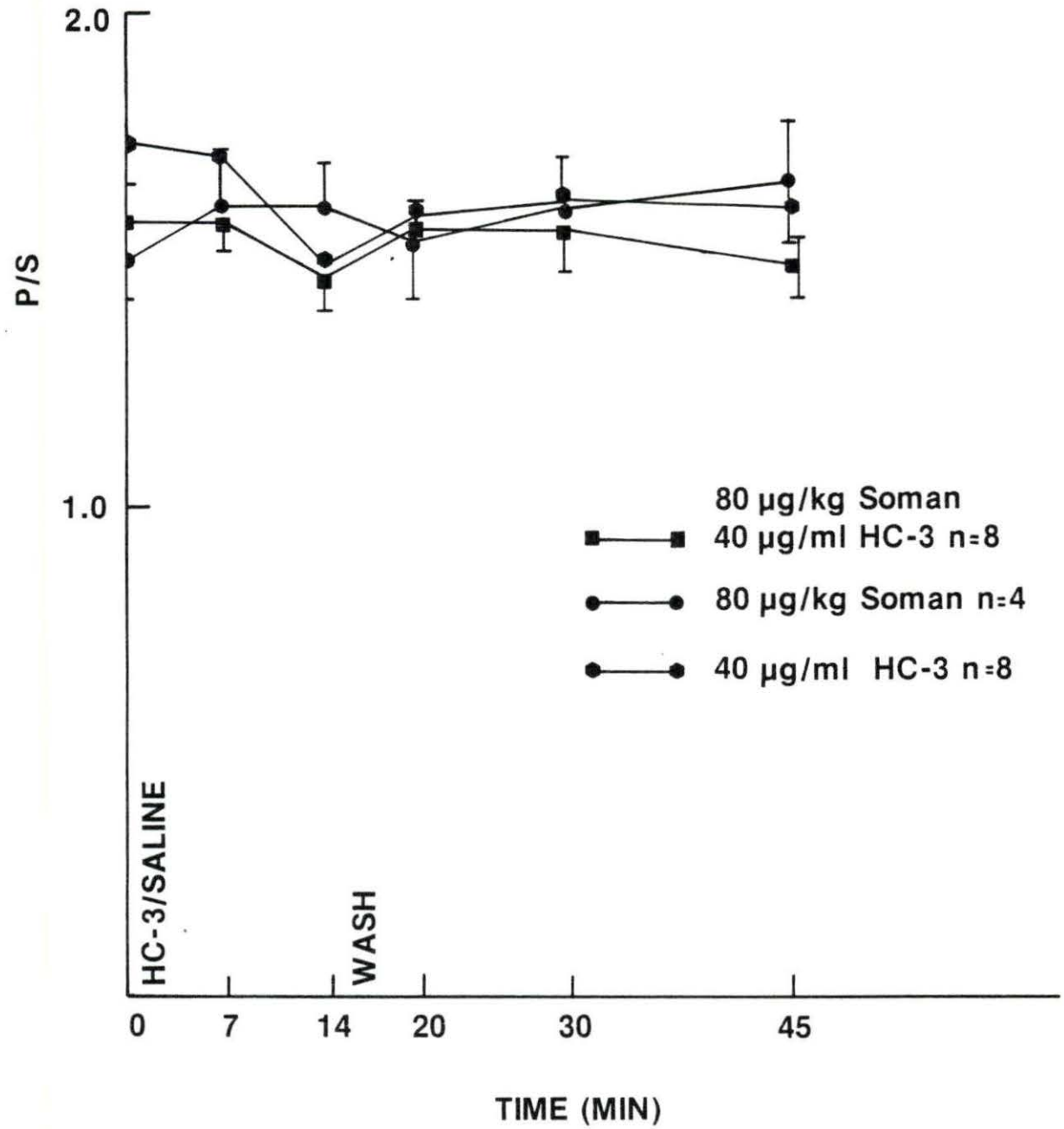

2.0
1.0
P/S
80 µg/kg Soman
40 µg/ml HC-3 n=8
80 µg/kg Soman n=4
40 µg/ml HC-3 n=8
HC-3/SALINE
WASH
0
7
14
20
30
45
TIME (MIN)

Fig. 14. HC-3 induces recovery from depression of T/S ratio in preparations from rats receiving soman (160 μg/kg)

The T/S ratio of preparations taken from rats receiving 160 μg/kg soman s.c. is depressed compared to control preparations. The rate of recovery of T/S ratio in preparations given 40 μg/ml HC-3 *in vitro* is greater than in preparations given saline *in vitro*. The graph shows the mean ± s.e.m. ▲ - non-soman pretreatment (control). ● , ■ - soman (160 μg/kg) pretreatment.

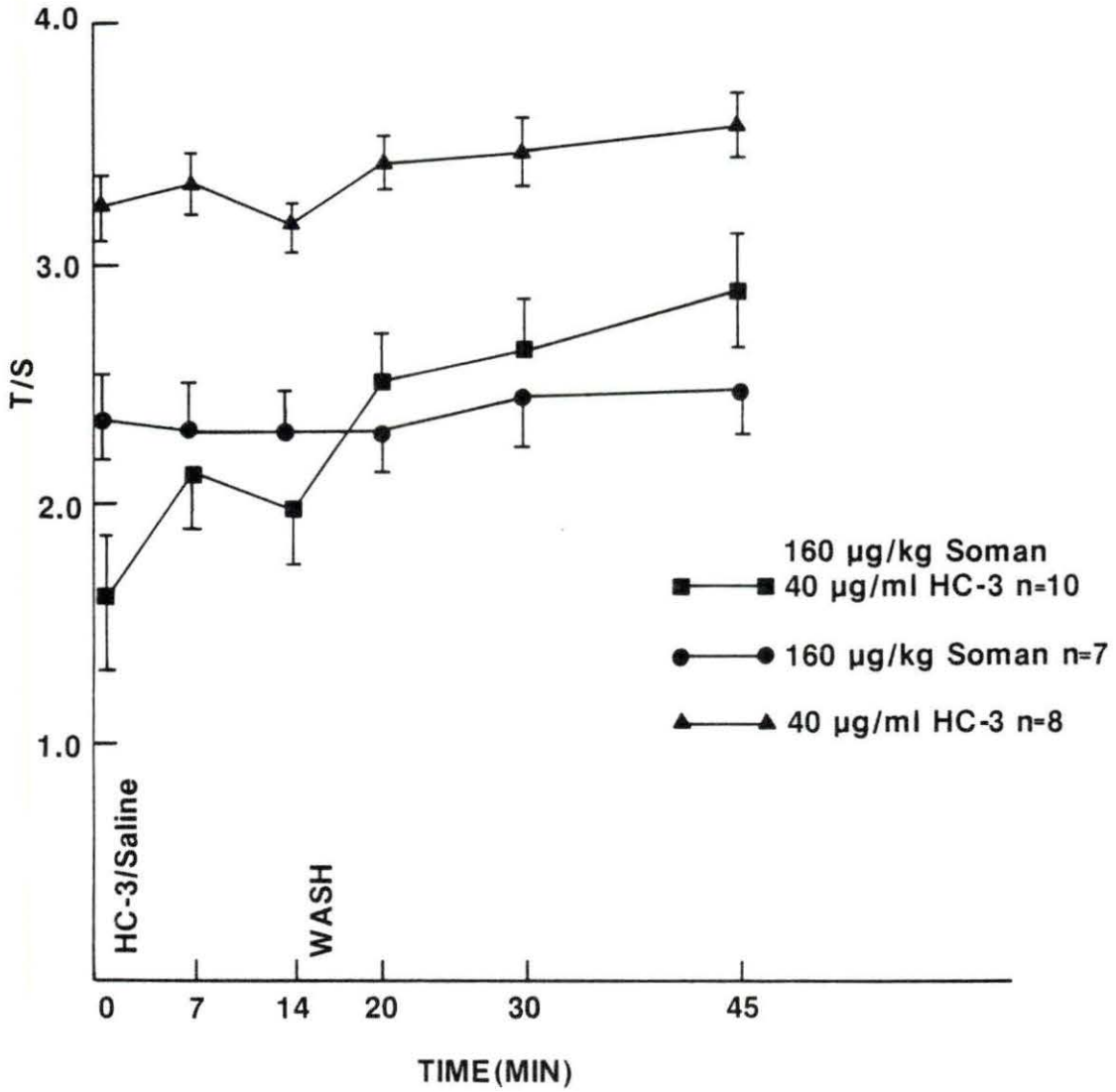
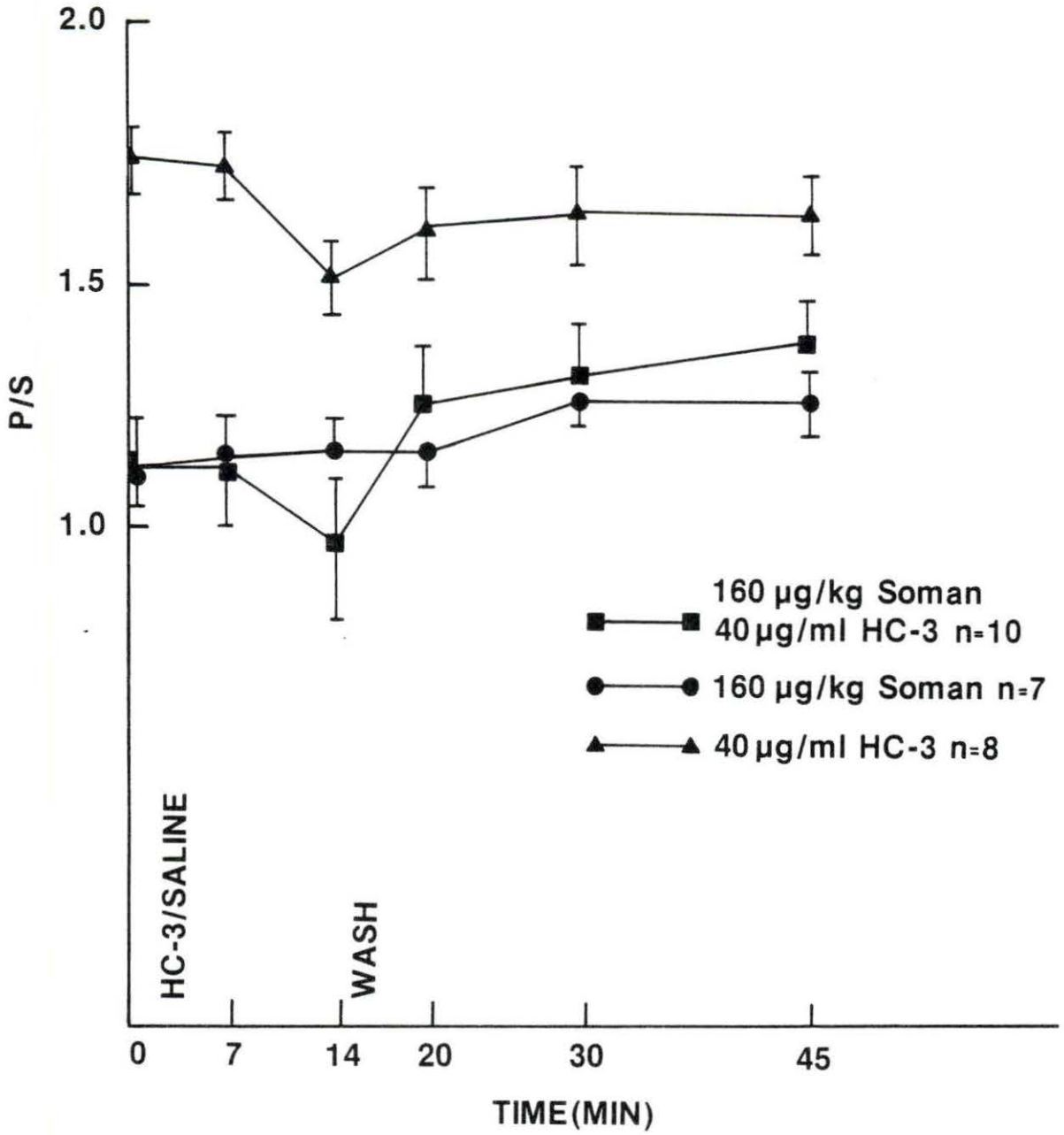

4.0
3.0
2.0
1.0
T/S
HC-3/Saline
WASH
0
7
14
20
30
45
TIME(MIN)
160 µg/kg Soman
40 µg/ml HC-3 n=10
160 µg/kg Soman n=7
40 µg/ml HC-3 n=8

Fig. 15. HC-3 fails to induce recovery from depression of P/S ratio in preparations from rats given soman (160 µg/kg s.c.)

The P/S ratio of preparations taken from rats given 160 µg/kg soman s.c. is depressed compared to controls. Neither HC-3 (40 µg/ml) nor saline given *in vitro* induces significant recovery from this depression. The graph shows the mean ± s.e.m. ▲ - non-soman pretreatment (control). ●, ■ - soman (160 µg/kg) pretreatment.


2.0
1.5
1.0
P/S
160 µg/kg Soman
40 µg/ml HC-3 n=10
160 µg/kg Soman n=7
40 µg/ml HC-3 n=8
HC-3/SALINE
WASH
0
7
14
20
30
45
TIME(MIN)

T/S ratio and P/S ratios increased (Figs. 14, 15), but only the increase in T/S ratio was statistically significant (Table 1). Saline did not affect the T/S ratio or the P/S ratio in preparations taken from rats receiving 160 μg/kg soman.

Table 1. The effect of HC-3 on the increase in T/S ratio in preparations taken from rats receiving 160 μg/kg soman or 320 μg/kg soman

Treatment	Increase in T/S between 0 and 20 min (mean + s.e.m.)	
	160 μg/kg soman	320 μg/kg soman
HC-3 (40 μg/ml)	0.291 ± 0.16**	1.278 ± 0.42*
Saline (0.5 ml)	-0.084 ± 0.12	0.306 ± 0.09

*p 0.05.
**p 0.01.

The T/S and P/S ratios of preparations taken from rats receiving 320 g/kg soman were further depressed. Exposure to HC-3 (40.0 μg/ml) for 6 min increased the T/S ratio (Fig. 16), but not the P/S ratio (Fig. 17). After 13 min exposure to HC-3, the increase in T/S ratio was partially reversed (Fig. 16), and P/S ratio was decreased (Fig. 17). When HC-3 was washed out, the T/S ratio further increased (Fig. 16), and the depression of the P/S ratio was reversed (Fig. 17). Saline did not affect the T/S or P/S ratios (Figs. 16, 17) in preparations from rats receiving 320 μg/kg soman.

Fig. 16. HC-3 increases rate of recovery from depression of T/S ratio in preparations from rats receiving soman (320 μg/kg)

The T/S ratio of preparations taken from rats receiving 320 μg/kg soman s.c. is depressed compared to control preparations. The rate of recovery of T/S ratio in preparations given 40 μg/ml HC-3 *in vitro* is greater than in preparations given saline *in vitro*. The graph shows the mean ± s.e.m. ⬢ - non-soman pretreatment (control). ●, ■ - soman (320 μg/kg) pretreatment.

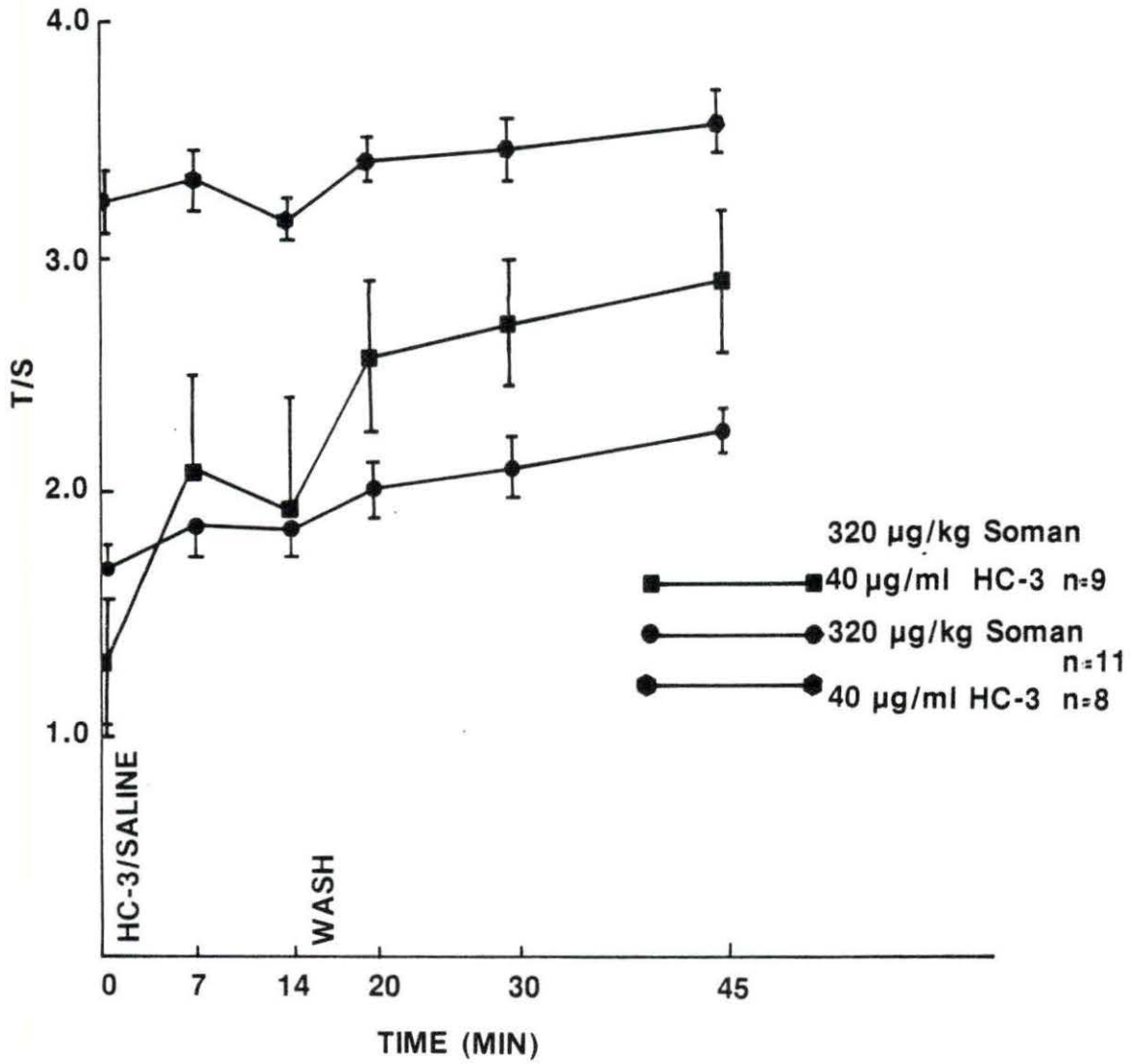
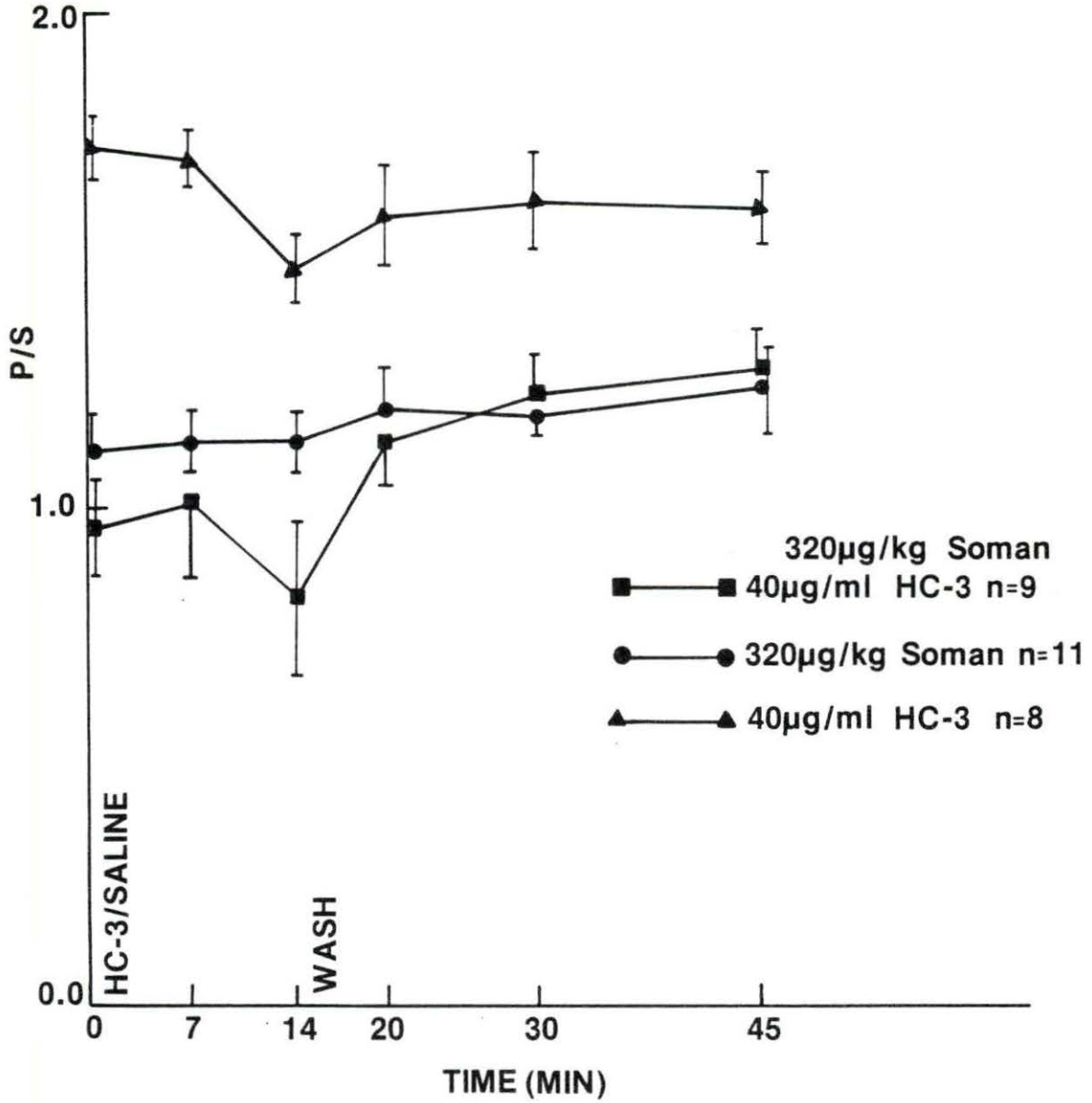

4.0
3.0
2.0
1.0
T/S
HC-3/SALINE
WASH
0
7
14
20
30
45
TIME (MIN)
320 µg/kg Soman
40 µg/ml HC-3 n=9
320 µg/kg Soman
n=11
40 µg/ml HC-3 n=8

Fig. 17. HC-3 fails to affect rate of recovery from depression of P/S ratio in preparations from rats given soman (320 µg/kg s.c.)

The P/S ratio of preparations taken from rats given 320 µg/kg soman s.c. is depressed compared to controls. Neither HC-3 (40 µg/ml) nor saline given *in vitro* induces significant recovery from this depression. The graph shows the mean ± s.e.m. ▲ - non-soman pretreatment (control). ●, ■ - soman (320 µg/kg) pretreatment.


2.0
1.0
0.0
P/S
HC-3/SALINE
WASH
0
7
14
20
30
45
TIME (MIN)
320µg/kg Soman
40µg/ml HC-3 n=9
320µg/kg Soman n=11
40µg/ml HC-3 n=8

HC-3 significantly increased the change in T/S ratio from the beginning of recording (0) to 5 min after HC-3 was washed out (20) in preparations from rats receiving either 160 μg/kg or 320 μg/kg soman (Table 1).

LD_{50} studies were performed using rats to compare the toxicity of HC-3 and a tertiary hemicholinium (t-HC-3). Using a dose range of 20-60 mg/kg t-HC-3, the LD_{50} was estimated to lie between 35 and 40 mg/kg (Table 2), when administered subcutaneously.

Table 2. Relationship between mortality of rats and dose of tertiary hemicholinium (t-HC-3) following subcutaneous injection

Dose of t-HC-3 (mg/kg)	No. of animals (n)	% Mortality
20	4	0
30	6	0
40	9	67
50	10	100
60	6	100

Using a dose range of 0.1-5.0 mg/kg HC-3, the LD_{50} was estimated to lie between 0.2 and 0.3 mg/kg (Table 3) when administered subcutaneously. Thus HC-3 was found to be over a hundredfold more toxic than t-HC-3.

Table 3. Relationship between mortality of rats and dose of hemicholinium-3 (HC-3) following subcutaneous injection

Dose of HC-3 (mg/kg)	No. of animals (n)	% Mortality
0.1	4	0
0.2	7	14
0.3	6	83
0.5	8	100
1.0	10	100
5.0	4	100

DISCUSSION

HC-3 causes a progressive reduction in contraction amplitude of the rat isolated phrenic nerve-diaphragm preparation at a stimulation frequency of 1.0 Hz. This action is thought to be due to HC-3 antagonism of high affinity choline uptake, thus reducing the amount of substrate available for ACh synthesis (Bowman, Hemsworth, and Rand, 1967). The recovery of muscle contraction amplitude in the presence of choline chloride, and the failure of HC-3 to induce neuromuscular blockade at 0.2 Hz (Fig. 2) provides further evidence that HC-3 blockade of contraction amplitude at 1.0 Hz is due to presynaptic mechanisms.

The reason for successive doses of 40.0 μg/ml HC-3 causing the preparation to become more sensitive to HC-3 is unclear. HC-3 is not thought to be an irreversible antagonist of choline transport (Yamamura and Snyder, 1973), yet HC-3 acts in a reversible manner only after a sensitization has occurred.

Chang and Rand (1960) and Hemsworth (1971) both reported that choline was unable to reverse neuromuscular blockade in the rat diaphragm in the presence of HC-3. These results (Fig. 3), which show that HC-3 can be antagonized by choline in the presence of HC-3, are in agreement with Takagi et al. (1970), who reported that at a stimulation frequency of 0.7 Hz, 10 μg/ml HC-3 was antagonized by 10 μg/ml choline. The dose-response relationship obtained for HC-3 (Fig. 3) is a typical example of a drug-receptor interaction, in which the binding of drug to receptor is reversible (Goldstein, Aronow, and Kalman, 1974). Antagonism of HC-3 by preadministered choline (Fig. 4) and the effect of preadministered choline

on the dose-relationship of HC-3 (Fig. 5) are a demonstration of properties of a competitive antagonist. Choline has been shown to be a competitive antagonist of HC-3 in whole animal toxicity (Schueler, 1955), at the cat superior cervical ganglion (McIntosh, Birks, and Sastry, 1956), and at the neuromuscular junction (Reitzel and Long, 1959a; Chang and Rand, 1960); thus the competitive antagonism of HC-3 by choline in the rat phrenic nerve-diaphragm is compatible with established data.

Thus, because neuromuscular blockade is frequency dependent, and because HC-3 is competitively antagonized by choline, the slow, progressive antagonism of contraction amplitude of the rat phrenic nerve-diaphragm preparation by HC-3 at 1 Hz stimulation occurs due to the inhibition of presynaptic uptake of choline at the neuromuscular junction. The most likely site of action is the sodium dependent high affinity choline transport system (c.f. Yamamura and Snyder, 1973). The blockade of choline uptake results in diminished ACh synthesis, and thus a reduced pool of ACh available for release.

Application of 50 Hz stimulation to the phrenic nerve caused tetanic contractions of the diaphragm (Fig. 6). Tetanic contractions occur because high frequency (50 Hz) stimulation of the phrenic nerve causes release of a large number of quanta of ACh which in turn stimulates the postsynaptic nicotinic receptors (Hobbiger, 1976). Administration of DFP causes single twitch contraction amplitude to increase, a phenomenon first observed by Van der Meer and Meeter (1956a) using 5.0 μg/ml DFP. In addition, Van der Meer and Meeter (1956a) also observed a subsequent reversal of increased single twitch amplitude in the presence of DFP.

The increase of single twitch contraction amplitude is thought to be due to cholinesterase inhibition causing a persistence of ACh, which in turn causes presynaptic stimulation (positive feedback) and thus repetitive firing of muscle. The subsequent decrease in contraction amplitude is thought to be caused by almost complete cholinesterase inhibition, leading to enough over-stimulation of the postsynaptic receptors by ACh that depolarizing-desensitizing block occurs (Hobbiger, 1976).

The reduction of tetanic contraction amplitude by DFP also occurs because of a depolarizing-desensitizing block induced by cholinesterase inhibition (Hobbiger, 1976). Cholinesterase determinations of rat diaphragms after DFP administration have revealed that tetanic blockade does not occur until cholinesterase activity is less than 15% of control (Barnes and Duff, 1953; Barstad, 1960; Hobbiger, 1976).

Administration of 1.0 μg/ml DFP caused a more pronounced decrease in tetanic contraction amplitude than prior administration of 2.0 μg/ml DFP (Figs. 6, 7, 8). This nondose-dependent action demonstrates that DFP is an irreversible antagonist of acetylcholinesterase, and that DFP (2.0 μg/ml) noncompetitively inhibits a certain fraction of the enzyme.

HC-3 antagonizes DFP induced tetanic blockade (Fig. 7, 8) by antagonism of presynaptic choline transport. Deprived of the necessary substrate, ACh synthesis is reduced, and this in turn causes reduced ACh release (Bowman & Marshall, 1972). DFP is antagonized when ACh release is reduced to an extent that it no longer accumulates in the synaptic cleft.

Post-tetanic potentiation is thought to primarily occur because of residual elevated calcium levels in presynaptic terminals (McIntosh and

Collier, 1976), causing residual elevated release of ACh. DFP induces post-tetanic depression by the mechanism of tetanic blockade, i.e., cholinesterase inhibition; and HC-3 antagonized DFP by the competitive inhibition of presynaptic choline transport as described above. After administration of 1.0 μg/ml DFP, post-tetanic potentiation did not recover to pre-DFP (Fig. 9). The reason for this is unknown.

Injection of 80 μg/kg soman s.c. into rats did not affect either amplitude of tetanic contractions (Fig. 12) or post-tetanic potentiation (Fig. 13), presumably because insufficient soman was distributed to the diaphragm to cause discernible blockade. However, many of the rats injected with 80 μg/kg soman (equivalent to 1 LD_{50}) displayed signs of soman poisoning, showing that the neuromuscular junction was not the primary focus of soman toxicity. Injection of 160 μg/kg and 320 μg/kg soman did cause significant depression of tetanic contraction amplitude (Figs. 10, 11, 14, and 16) as measured by the T/S ratio, and also depressed post-tetanic potentiation (Figs. 10, 11, 15, and 17). Depression of tetanic contraction amplitude was observed by French, Wetherell, and White (1979) with 40 nM soman in vitro. In their study, 40 nM soman caused almost complete tetanic blockade, and cholinesterase activity was found to be 6% of control. The mechanism of action of soman in this study is presumed to be irreversible cholinesterase inhibition at the neuromuscular junction, as other studies have correlated soman-induced tetanic blockade with cholinesterase inhibition in the rat diaphragm and other neuromuscular preparations (Dirnhuber and Green, 1978; French, Wetherell, and White, 1979).

However, the spontaneous recovery from tetanic blockade, seen after 320 μg/kg soman, is in contrast with French, Wetherell, and White (1979) who found a permanent blockade with soman *in vitro*. Reasons for spontaneous recovery are not clear, although it is possible that soman action is not completely due to cholinesterase inhibition, and that any other effects (probably postsynaptic) are reversible. Another possibility is that the sensitivity of the ACh postsynaptic receptors are gradually adjusted to adapt to excessive ACh stimulation. However, these hypotheses are, as yet, untested.

HC-3 antagonism of soman depression of T/S ratio is due to presynaptic antagonism of choline transport. HC-3 thus antagonizes soman by the same mechanism as it antagonized DFP. The mechanism by which HC-3 causes a transitory depression of both T/S and P/S ratio while HC-3 is in contact with the diaphragm (Figs. 14, 15, 16, and 17) is unknown. The reason why HC-3 induced the recovery of P/S ratio depressed by DFP, but not P/S ratio depressed by soman is also unclear.

The results of LD_{50} studies revealed that the tertiary hemicholinium (t-HC-3) was approximately one-hundred times less toxic than HC-3, however, these two drugs are equipotent in minimum effective dose at the rat isolated phrenic nerve-diaphragm (Grieve and VanMeter, unpublished data). Possible reasons for this discrepancy include differences in susceptibility to enzymatic degradation, or differences in protein binding; it should be noted that nothing is at present known about the biochemical pharmacology of t-HC-3.

In summary, HC-3 causes a delayed and progressive inhibition of single twitch contractions of the rat isolated phrenic nerve-diaphragm preparation; and because this inhibition is frequency dependent and is antagonized by choline, HC-3 acts by antagonizing presynaptic choline transport and thus causes diminished ACh synthesis. By the same mechanism of action, HC-3 antagonizes depression of tetanic contraction amplitude and depression of post-tetanic potentiation caused by organophosphorous cholinesterase inhibitors soman and DFP in the rat isolated phrenic nerve-diaphragm preparation.

ABSTRACT

The progressive reduction of muscle single twitch amplitude by HC-3 and the antagonism of this response by choline were examined in the rat isolated phrenic nerve-diaphragm preparation. HC-3 antagonizes muscle contractions in response to 1.0 Hz but not 0.2 Hz phrenic nerve stimulation, indicating a frequency dependent action as reported in the literature.

The effect of HC-3 to 1.0 Hz stimulation is antagonized by preadministered and postadministered choline chloride (8.0 μg/ml), at HC-3 doses of 4.0, 6.0, and 8.0 μg/ml; but choline fails to antagonize HC-3 at HC-3 doses of 10.0, 12.0, and 16.0 μg/ml. As HC-3 action is frequency dependent and is antagonized by choline, it is proposed that HC-3 acted by competitive inhibition of the presynaptic sodium dependent high affinity choline transport system.

The response of the preparation to 50 Hz tetanic stimulation and the effect of DFP and soman on this response also was studied. DFP (2.0 μg/ml) *in vitro* causes depression of tetanic contraction amplitude and post-tetanic depression, as does a subsequent administration of 1.0 μg/ml DFP *in vitro*. HC-3 (40.0 μg/ml) prevents tetanic blockade and post-tetanic depression in response to 1.0 μg/ml DFP.

Soman was administered subcutaneously to rats at doses of 80.0, 160.0, and 320.0 μg/kg, and the phrenic nerve-diaphragms were removed for *in vitro* investigation. Preparations removed from rats given 80.0 μg/kg soman did not differ from controls given saline. In preparations from rats given 160.0 μg/kg or 320.0 μg/kg soman, tetanic blockade and post-tetanic

depression were seen. HC-3 (40.0 μg/ml), administered *in vitro*, significantly increased the rate of recovery of preparations from tetanic blockade, but did not significantly promote recovery of post-tetanic depression.

It is proposed that DFP and soman caused tetanic blockade and post-tetanic depression by cholinesterase inhibition, leading to ACh accumulation, and HC-3 antagonized DFP and soman by blockade of presynaptic choline transport. HC-3 was found to have a subcutaneous LD_{50} of approximately 0.25 mg/kg in rats. A tertiary hemicholinium (t-HC-3) was synthesized as a potential antagonist of organophosphates. This compound was found to have a subcutaneous LD_{50} of approximately 37 mg/kg in rats.

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APPENDIX A

Composition of Krebs' solution (g/litre)

NaCl	6.9
KCl	0.35
$MgSO_4$, FH_2O	0.29
KH_2PO_4	0.16
Glucose	2.0
$NaHCO_3$	2.1
$CaCl_2$	0.28

APPENDIX B

1. Dose cycle for HC-3/Choline studies

X μg/ml HC-3 until T_{50}

WASH

8.0 μg/ml Choline chloride until recovery

X μg/ml HC-3 until T_{50}

WASH

8.0 μg/ml choline chloride until recovery

WASH

Doses of HC-3 were 4.0, 6.0, 8.0, 10.0, 12.0 and 16.0 μg/ml, given in that order.

2. Protocol for HC-3/DFP antagonism studies

Time (min)	Procedure
-02.00	50 Hz stimulation
00.00	2.0 µg/ml DFP
07.00	50 Hz stimulation
14.00	50 HZ stimulation
15.00	WASH
20.00	50 Hz stimulation
30.00	50 Hz stimulation
45.00	50 Hz stimulation
60.00	1.0 µg/ml DFP
67.00	50 Hz stimulation
74.00	50 Hz stimulation
75.00	WASH
80.00	50 Hz stimulation
90.00	50 Hz stimulation
105.00	50 Hz stimulation

3. Protocol for HC-3/soman antagonism studies

Time (min)	Procedure
00.00	50 Hz stimulation
01.00	40.0 µg/ml HC-3 or 0.5 ml saline
07.00	50 Hz stimulation
14.00	50 Hz stimulation
15.00	WASH
20.00	50 Hz stimulation
30.00	50 Hz stimulation
45.00	50 Hz stimulation