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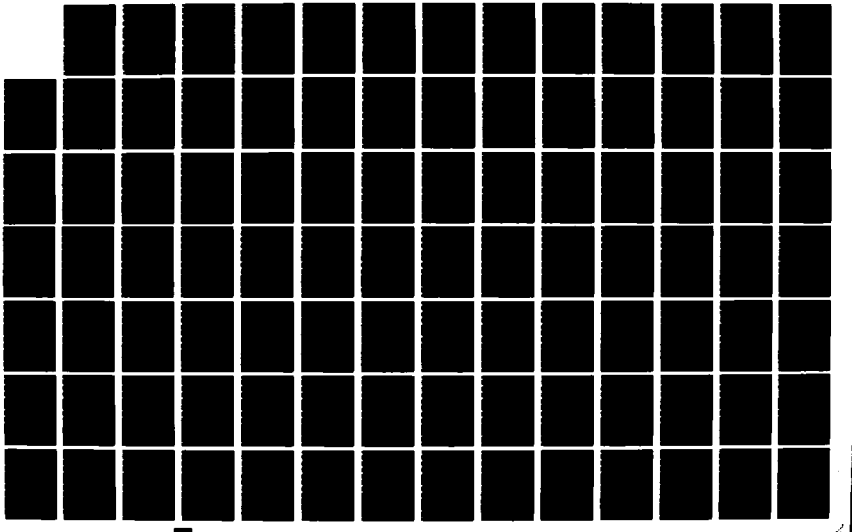

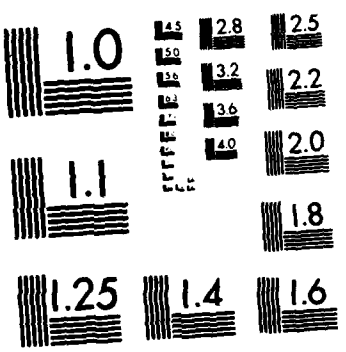

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EFFECTS OF CHEMICAL AGENTS ON THE CHOLINERGIC NEUROTRANSMITTER SYSTEM: MECHANISMS OF ADAPTATION

ANNUAL SUMMARY REPORT

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These differences have significant implications for effects of direct cholinergic agonists and compounds, e.g. anticholinesterase, which act as indirect agonists by increasing acetylcholine levels in brain and other tissues.

The results of experiments using the anticholinesterase, DFP, showed that a significant effect of chronic depression in acetylcholinesterase (AChE) activity is disinhibition of acetylcholine (ACh)- evoked release from presynaptic neurons, elevating ACh levels in brain. When viewed within the general context of knowledge about adaptive processes within the cholinergic system, these results suggest that the elevated ACh levels could initiate processes involved in a significant downregulation of mAChRs that is maintained during the continued decrease in AChE activity induced by the anticholinesterase. The modulation of mAChRs has been shown to occur concomitantly with adaptive changes in behavioral and physiological functions of the total, integrated organism.

The results of the present experiments have relevance for the wide variety of situations in which individuals or groups are exposed occupationally or adventitiously to organophosphous anticholinesterase compounds.

SUMMARY

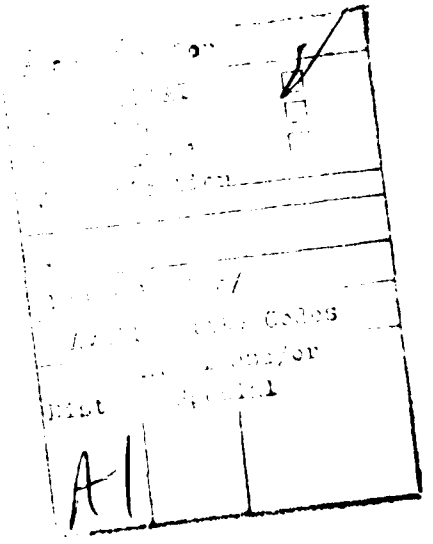
The present research program was designed to expand existing knowledge about behavioral and neurochemical adaptation during chronic exposure to chemicals which affect the cholinergic neurotransmitter system and also about effects of abrupt withdrawal from such exposure. During the first year of the project, experiments were completed involving three different types of chemical agent: an anticholinesterase, DFP; a cholinergic agonist, oxotremorine; and two oxotremorine mustards, BM 123 and BM 130. The studies were of four major kinds: pharmacological, neurochemical, behavioral and physiological. The extensive nature of the results obtained permit, reference in an abstract to only a few major findings.

The general pharmacological investigations were directed primarily toward the mustard analogs of oxotremorine and used in vitro and in vivo assays. The two compounds, BM 123 and BM 130, were found to have similar properties, differing mainly in their chemical kinetics in aqueous solution and in their potency. Both central and peripheral muscarinic effects of relatively short duration were observed after intravenous injection and were followed by a long-lasting resistance to cholinomimetic agents. When the two compounds were allowed to cyclize before injection, only peripheral effects were noted. All these effects were prevented by premedication with atropine. Studies of specificity for binding to muscarinic receptors (mAChRs) found BM 123 to be 175-fold and BM 130, 8-fold more selective than the cholinergic agonist, carbachol.

Neurochemical studies also focused primarily on the oxotremorine mustards, using both in vitro and in vivo preparations. Incubation of tissue homogenates with various concentrations of BM 123 and BM 130 caused an irreversible and persistent inhibition of [^{3}H]QNB binding. The kinetics of this binding was examined in detail. Other binding experiments showed that it is possible to alkylate the different agonist states of mAChR selectively with BM 123. A multiple dose regimen for acute administration in vivo was developed which occluded total mAChR in various brain areas and in heart by 90% or more. The time course of reappearance of receptors following this regimen was similar in the brain regions, with a half-time of 26 hr. The effects of chronic administration of BM 123 were to produce dramatic differences in recovery rates following withdrawal: heart recovered most rapidly, then longitudinal muscle, with brain the slowest. A major experiment with DFP, using the myenteric plexus preparation, gave results which suggest that pre- as well as postsynaptic mAChRs are downregulated during chronic exposure, the effects being adaptive in the case of postsynaptic changes (e.g., as reflected in development of tolerance) and non-adaptive at the presynaptic site.

Behavioral and physiological studies were carried out in three series of experiments using the four chemical agents (BM 130, BM 123, DFP, and oxotremorine) and an extensive battery

of assays. Intravenous injections of BM 130 were followed by central and peripheral signs which were consistent with those produced by cholinergic agonists, e.g., oxotremorine. Acute effects of short duration were seen in core body temperature (hypothermia) and in nociception (hypoalgesia). There were no acute effects on learned behaviors, conditioned avoidance and fixed ratio responding, which were under control of two quite different types of reinforcement. Observations of prolonged effects were consistent with the hypothesis that acute effects of BM 130 are followed by sustained resistance to cholinergic agonists -- e.g., responding to oxotremorine challenges was suppressed -- and that the protection involves central mechanisms. Comparisons of the effects of BM 123 and oxotremorine confirmed these observations, i.e., that the mustard form produces a sustained change in receptor-mediated events which would be expected from an irreversible ligand. More detailed results suggest widely differing sensitivity of different neural circuits to interference by BM 123, differences that could be due to the stabilizing effect of neural feedback loops or to the presence of a large receptor reserve at some sites. Results from the third series of experiments, designed to study effects of abrupt withdrawal following chronic treatment with DFP, are still undergoing detailed analyses. Some preliminary observations are given in the body of the report.



FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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1. STATEMENT OF THE PROBLEM

Defense against the possibility that military personnel and sectors of the general population may be exposed to chemicals which affect the cholinergic neurotransmitter system depends upon an understanding of the plasticity of the system to chronic low level exposure as well as acute exposure. The proposed research is aimed at expanding present knowledge about behavioral and neurochemical adaptation to these agents and to withdrawal from chronic exposure. The major drug groups to be studied are organophosphate and carbamate anticholinesterases (anti-ChEs), muscarinic agonists and antagonists and inhibitors of high affinity transport of choline, the process believed to be rate-limiting in acetylcholine (ACh) synthesis. A series of neurochemical and behavioral probes will be used to establish the time course and mechanisms of tolerance development, including cross-tolerance or sensitization to challenge doses of other agents. The proposed program, by clarifying the mechanisms of action of compounds affecting the cholinergic system, can provide a rationale for the design and development of drugs of preventive and therapeutic value and regimens for their most effective use. Such knowledge may also be expected to help alleviate conditions underlying pathological states involving malfunction of the cholinergic system.

2. BACKGROUND

2.1 CHOLINERGIC MECHANISMS IN ADAPTIVE PROCESSES

There is ample evidence to implicate cholinergic mechanisms in a wide variety of adaptive processes in living organisms as changes occur in their internal and external environments. Such processes are evident both neurochemically and behaviorally; concomitant variations between the two suggest that they are related and that the latter is a consequence of variations in the former, i.e., that the cholinergic system provides substrates for behavior (1). The integrated actions of the neurochemical and behavioral systems provide the plasticity which characterizes the adaptive processes. The systems concept of living organisms proposes that adaptation normally occurs within the limits of such plasticity (2). When limits are exceeded, malfunctions occur, as in cases of spontaneous states of neuropsychiatric disturbance and of perturbations caused by exposure to chemical agents.

Among the adaptive processes are two which are functions of repeated or chronic exposure to a drug or chemical agent: tolerance and withdrawal rebound. The course of these processes can be studied (a) by measuring changes in neurochemical or behavioral variables at various times during chronic exposure or (b) by measuring effects on such variables of "challenges" by other drugs or agents. By concurrent measurement of neurochemical and behavioral variables, hypotheses about relations between the two can be tested. Other hypotheses about the order of such relations, i.e., which is the antecedent and

which the consequent, can be studied by direct manipulation of one class of variable while measuring the other. All of these logical approaches have been used in studying tolerance and can be as readily applied to the investigation of withdrawal phenomena. In the present research program, manipulation of the cholinergic system provides the independent variable; measures of concomitant neurochemical physiological and behavioral effects, the dependent variables.

2.2 MANIPULATING THE CHOLINERGIC SYSTEM EXPERIMENTALLY

Cholinergic function may be altered experimentally at several points, although agents acting at any one of these are seldom specific, and generally act also at others.

Inhibition of high affinity Ch transport. Hemicholinium (intraventricularly) both depletes and inhibits synthesis of ACh and produces characteristic behavioral effects. Inhibition of synthesis has also been demonstrated in isolated and perfused preparations (3,4) and in synaptosomes, where the inhibition of incorporation of labeled pyruvate and labeled Ch into ACh has been shown to be linearly related (5). Hemicholinium has been shown to be an extremely potent inhibitor of high affinity choline transport, to which the depletion and inhibition of synthesis are usually ascribed. Many compounds apparently behave as poor substrates of the high affinity carrier (6) and may be acetylated in intact tissues or synaptosomes. Some of the acetylated compounds are released under stimulation and therefore behave as false transmitters (7), which have been used to analyze the nature of the coupling between transport and acetylation (6) and the kinetics of synthesis, turnover and release (8).

Interference with supply of Acetyl-CoA. This interference may be accomplished by hypoxia (9), glucose restriction, inhibition of pyruvate transport (10) or inhibition of pyruvate dehydrogenase (11). Any of these measures will also interfere with metabolic processes of all cells. It may be significant, however, that pathological states involving any of these steps may produce effects referable to cholinergic hypofunction (12), and hence may be susceptible to therapeutic amelioration based on this mechanism. If the supply of acetyl-CoA is subject to regulatory control, this point of potential pharmacological attack remains to be exploited.

Inhibition of choline acetyltransferase (CAT). CAT inhibitors are clearly a potentially interesting class of compounds, and a number of potent inhibitors of this enzyme have been reported (13), some of which are quite specific and have very little effect upon cholinesterases. Two of these (naphthylvinylpyridine and 3'-chlorostilbazole) have been investigated pharmacologically in some detail (14), and these studies have led to the important conclusion that CAT could not be rate-limiting, since levels and turnover of ACh in rat and mouse brain were essentially unaffected at the expected doses, despite the fact that brain concentrations of one of the

inhibitors reached levels up to 100 times the *in vitro* I_{50}. A different type of CAT inhibitor has recently been described (15) which is more water-soluble and therefore less likely to be protein bound than earlier inhibitors.

Interference with release of ACh. The mechanisms of release and the factors controlling it are still not well understood. The literature is complicated by the fact that the rate of release *in vivo* and in many preparations *in vitro* may be influenced not only by a direct effect on the release mechanism but by indirect actions involving other modulators or even neuronal loops. The most logical way to resolve most of these ambiguities is by parallel studies on several experimental models of graded complexity.

Interference with the inactivation of ACh. For ACh to be effective as a neurotransmitter, it must be released within time limits required for the normal response characteristics of the biological units upon which it acts and then be removed. Acetylcholinesterase (AChE) is responsible for hydrolyzing ACh which is released from the nerve terminal. Assays by gas chromatography-mass spectrometry GCMS (16) of homogenates from whole brain or from brain regions of rats have shown that ACh is [elevated] after acute injection of the anti-ChE, diisoprophyl flourophosphonate (DFP), and remains so throughout a period of chronic administration (17). By leading to accumulation of endogenous ACh at its receptor sites, the anti-ChE produces effects which are due to excessive stimulation of the receptors; i.e., the anti-ChE acts as an indirect agonist.

Effects at cholinergic receptors. Many classical agents are known which exert agonist or antagonist effects at cholinergic receptors, some of which are well known, extremely potent and commonly used both experimentally and clinically. The oxotremorine analogs used in the present study are unique in several respects; they are not only exceedingly potent but relatively specific for the central nervous system (CNS), and include partial agonists and irreversible ligands.

Among these several possibilities, the present research program is focused on the last two.

2.3 ANTICHOLINESTERASES

Anti-ChE agents have received much attention from researchers and the general public because of their extensive applications as toxic agents, particularly in the form of agricultural insecticides. Reports of human occupational or adventitious exposures describe general symptoms which include: loss of discrimination performance, difficulty in concentration and expressing thoughts, confusion, disorientation, and signs of anxiety and depression. Early experiments (1) using animal models concluded that exposure to anti-ChEs produced differential effects on behavior, some behavior patterns being affected and others not. Behaviors affected involved the extinction of old

responses which were no longer appropriate in coping with new environmental demands. Dose-effect data revealed a critical brain AChE activity level at about 45% of normal, below which the behavior was significantly affected. These behaviors were considerably more sensitive to effects of anti-ChEs than were other pathological signs. Such observations have been replicated in many more recent studies of a variety of anti-ChE agents, using different routes of entry and more sophisticated behavioral analyses. Improved techniques for neurochemical analyses of effects of anti-ChE agents on the cholinergic system have been applied in the investigation of relations between neurochemical and behavioral variables. The search for mechanisms of action involved in the development of behavioral tolerance to chronic decreases in AChE activity illustrates the direction in which research is now proceeding (18).

Observations involving both human subjects and animal models show that tolerance develops during chronic exposure to ChE inhibitors. Traditionally tolerance has been defined in terms of three major characteristics: an acute change in the criterion variable, a diminution in the effect with repeated administration of a fixed dose, and reinstatement of the original effect by an increase in dose. To these may be added the fact that tolerant subjects react quite differently than non-tolerant subjects to challenges by other chemical agents. Results of investigations of chronic exposures to anti-ChE compounds with different chemical structures have been sufficiently similar to warrant a general summary of their effects. Early parasympathomimetic signs of reduced ChE activity decrease with chronicity of treatment and disappear even as the enzyme activity continues to be depressed, as evidenced in serum, red blood cells and brain. Measurements of a wide variety of innate and acquired behaviors have shown that the same chronic treatment leads to differential effects; i.e., some behaviors show tolerance and others do not. For those variables that do develop tolerance, there are limits when exposures are sufficiently high to exceed the plasticity of the system and to produce gross pathological signs and incapacitation. Below these limits, magnitude of acute effects and duration of exposure (before tolerance is complete) are dose dependent (19). Studies in which subjects who have recovered to pretreatment behavioral baselines have been "challenged" with other agents make it clear that there may remain long-lasting biochemical effects in the absence of overt symptoms.

Several hypotheses have been proposed about the neurochemical mechanism(s) of action underlying tolerance development to anti-ChE agents. Among those eliminated after tests of their validity are: non-specific metabolic changes, end-product inhibition of ACh synthesis, and neurochemical redundancy with activity shunted into another, non-cholinergic pathway (20). The possibility that reduction in AChE activity could induce changes at presynaptic subcellular levels has been studied using *in vitro* and *in vivo* preparations. The *in vitro* studies have involved the use of techniques for subcellular fractionation and for integrated GCMS to measure endogenous and

tracer variants of Ch and ACh in synaptosomes prepared from the brains of rats at various stages of tolerance development (21). No statistically significant differences were found among treatment groups in the total concentration of ACh or Ch, the synthesis of ACh, or the high affinity transport of Ch. In the in vivo studies, rats at the same stages of tolerance were given pulse intravenous injections of [$^2H_4$]-Ch 1 min before death by microwave irradiation of the head. ^{4}Homogenates from the whole brain or from regions were assayed by GCMS. Significant increases (15% above control values) in total brain ACh were seen 4-48 hr after an acute injection and after 1-22 days of chronic administration. Total brain Ch did not vary concomitantly with the development of tolerance: levels were significantly decreased for 2-24 hr and were significantly greater than control values 10 and 22 days after chronic administration. No changes were seen in ACh synthesis or Ch uptake, indicating that the behavioral tolerance could not be due to end-product inhibition. Psychopharma- cological evidence suggested that the more probable mechanism(s) of tolerance to anti-ChE compounds might involve changes in muscarinic receptors. Direct evidence was first obtained by Schiller (22) and has now come from several other investigators (23, 24, 25) that postsynaptic mAChRs are indeed involved: tolerance development is associated with a decrease in numbers of postsynaptic mAChRs.

2.4 CHOLINERGIC AND ANTI-CHOLINERGIC AGENTS

Tolerance has been shown to develop during chronic administration of such agonists as oxotremorine. The studies are limited in number and in the variety of behaviors measured. Early experimentation showed tolerance developing to tremorine, but found no cross-tolerance to oxotremorine (26, 27). Later investigations observed acute tolerance development in as little as a few hours during treatments of 1-3 doses of pilocarpine, tremorine and oxotremorine (28, 29). Curves for such measures as salivation, tremor, hypothermia and motor coordination are reported to shift in parallel to the right on chronic exposure to oxotremorine, with no significant differences in amounts of receptor or its affinity (30). A more recent report in which chronic oxotremorine treatment was administered via an indwelling i.v. cannula describes both development of tolerance and changes in cholinergic neurochemistry (31). The former was observed in such symptoms as salivation, lacrimation and tremor and in measures of hypothermia and rotarod performance; concomitant changes, i.e., a decrease in number of mAChRs, occurred in some, but not all, of the aspects of brain cholinergic function studied. The authors of this report stated that behavioral tolerance seemed develop before significant alterations in Quinuclidinyl Benzilate (QNB) binding or CAT activity occurred. This observation suggests the possible involvement of additional mechanisms in the development of tolerance to oxotremorine. Similar observations have been made in cross-sectional studies of mAChR changes accompanying development of behavioral tolerance to the indirect agonist, DFP (22). More recent studies of ACh levels and dynamics in rat brain have led to the conclusion that,

under conditions of increased levels of ACh, it is not implausible that adaptive changes in receptor concentrations could result at both pre- and postsynaptic sites (17). The present limited state of knowledge about tolerance to cholinergic agonists and the mechanisms underlying this tolerance has led to the design of the present experiments, which will (a) expand information about the extent of susceptible behavior and (b) investigate presynaptic events, i.e., ACh synthesis and release, which may result from effects of chronically elevated levels of cholinergic function mediated via autoreceptors.

Effects on behavior of healthy human volunteers of exposure to anticholinergics have led to the concept of a "central anticholinergic syndrome (32)." Symptoms described have include hallucinations, confusion, incoherence, impaired memory and disorientation. Effects on behavior of acute administration of anticholinergics were observed in a number of experimental studies using animal models. As with drugs generally, the nature of the effects has been shown to be dependent upon the behavior (task) measured, and the efficacy with which they are acquired; i.e., effects are differential, not behaviors affecting all behaviors indiscriminately (18, 33). It is also the case that particular behaviors may be affected similarly by both cholinergic and anticholinergic agents (34). Relatively few experimental studies of behavioral effects of chronic administration of anticholinergic agents have appeared. Tolerance to the belladonna drugs occurs in man to a limited extent, e.g., patients with Parkinsonism may eventually receive daily doses of atropine or scopolamine that, if given to the uninitiated, would result in poisoning. That adaptation (tolerance) to repeated doses of anticholinergics can also occur in normal human subjects has been demonstrated experimentally (35). Limitations on research with such subjects has required the focusing of attention on animal models. Reports of diminished responsivity on a passive avoidance task to a second administration of scopolamine suggest that tolerance to such agents could develop rapidly during chronic exposure. Experiments involving longer periods of chronic treatment have reported the occurence of tolerance development in a variety of behaviors including locomotor activity, exploratory behavior, several schedules of operant responding, conditioned avoidance behavior, visual discrimination, maze performance, and behavior under conditions requiring multiple schedule responding. Early studies of tolerance indicated that neurochemical changes could take place without readily recognizable overt signs. In such instances the possibility that adaption had occured could be tested by "challenging" the system with another cholinergic or anticholinergic agent. For example, following a chronic regimen of scopolamine, administration of pilocarpine produced a supersensitivity of the hypothermic response (36). It is also significant that adaption to chronic scopolamine may include changes primarily of a behavioral rather than neuropharmacological origin, "behaviorally augmented" tolerance (37, 38). For example, administration of scopolamine before a trial prevents complete learning; after a learning trial it

appears to interfere with neither learning nor performance and allows the development of tolerance to pretreatment levels of behavior.

2.5 OXOTREMORINE ANALOGS

Although for purposes of comparison, the present research program will study tolerance development as it is evidenced in effects of chronic administration of certain of the well-known cholinergic and anti-cholinergic agents, special interest is in a series of oxotremorine analogs which are not generally available for research. These compounds (synthesized by Richard Dahlbom, Bjorn Ringdahl and their colleagues at the University of Uppsala, Sweden) (39) vary from strong-to-partial agonists to strong antagonists. Research in collaboration with Dahlbom has proceeded to the point where behavioral as well as neuropharmacological experiments are feasible and have begun. These compounds provide means of manipulating the function of the cholinergic system which have significant advantages. For example, the study of structure-activity relations is possible, using molecules which are basically similar but differ in particulars deliberately chosen prior to their synthesis. The availability of both tertiary and quaternary forms enables comparisons between peripheral and central effects without major intervention in the integrity of the organism via cerebroventricular or other routes. As with all new compounds, it is necessary to study their effects in vitro and in vivo, at subcellular, cellular and tissue levels and, finally, on physiological and behavioral assays.

3. APPROACH TO THE PROBLEM

The general strategy used in approaching the problem is based upon the concept of the "integrated organism." To cope with their ever-changing environments, living organisms depend upon interactions among all their basic properties: morphological, biochemical, electrophysiological and behavioral. Changes in any one of these may be reflected in changes in one or more of the others. When chemical agents enter the body through any route, their effects must be regarded as ultimate consequences of physiochemical interactions between the agent and functionally important molecules in the organism. Such interactions begin a series of effects which may have, as an end point, significant consequences for behavior, for the capability of the organism to make normal adjustments to the demands of its environment.

Based upon this general concept, the present research program undertakes a coordinated approach in which investigators with special capabilities range from organic chemists with the skills to synthesize chemical compounds of a desired structure to psychopharmacologists with expertise in the measurement of behavior. They include neurochemists, neuropharmacologists and specialists in electronics. By coordinating such knowledge and

skills, it is possible to study a particular compound throughout its entire course of action.

Putting this general approach to work, several specific hypotheses were tested during the first year of the program. As discussed above, the independent variable in each of these has consisted of pharmacological manipulations of the cholinergic system. Three different types of chemical agents were used: an anti-ChE, DFP; a cholinergic agonist, oxotremorine; and two oxotremorine mustards, BM 123 and BM 130. The inclusion of soman was delayed by the renovation of the laboratory to meet the requirements of the Facility Security and Safety Plan, but preparations have been made to enter it into the ongoing program in June 1984. The dependent variables measured are described in detail below. Effects of acute and chronic administration of the agents and their abrupt withdrawal were measured.

4. METHODS AND RESULTS

The paragraphs that follow report results of research during the first year of the contract. During that period the full range of capabilities of our laboratories were involved. This included: synthesis of new organic compounds, study of the formation and decomposition of their biologically active forms, determination of their neurochemical and pharmacological properties and assays of their behavioral and physiological effects. The compounds involved have promise for use in "protection" against exposure to antiAChE agents, incurred adventitiously or occupationally. The first year's research program also included experiments designed to study effects of the antiChE, DFP.

The following presentation is organized in two major sections, each with several subsections. The major sections indicate the primary thrusts of the overall research program: "Neurochemical and Pharmacological Studies"; "Behavioral and Physiological Studies". The subdivisions indicate more specifically the particular directions for experimentation during Year 1. It is important to understand that, within our overall plan, studies reported in both major sections were integrated in a manner which maximized the transfer of information among experiments. The section, "Neurochemical and Pharmacological Studies", reports results of a series of *in vivo* and *in vitro* experiments designed to determine the pharmacological characteristics of two mustard anaolgs of oxotremorine and their possible effects in providing long-lasting resistance to ("protection" against) both direct and indirect cholinergic agonists (e.g., anticholinesterases). This section also summarizes results of other *in vivo* and *in vitro* experiments concerned with vital steps in the mechanism(s) of action of the oxotremorine analogs, i.e., the formation and decomposition of aziridinium ions; their effects on the binding properties of muscarinic receptors in brain, heart and muscle tissues. The second major section, "Behavioral and Physiological Studies", present results relating these neurochemical and pharmacological

processes to the capabilities of the total, integrated organism to maintain or to regain it normal functioning during manipulation of its cholinergic system. The capability to adapt to such manipulation is essential if the organism is to survive and to cope with the requirements of the physical and psychosocial environments within which it must operate.

4.1 NEUROCHEMICAL AND PHARMACOLOGICAL STUDIES

4.1.1 Synthesis of BM 123, BM 130 and their hydrolysis products

The chemical structures and transformation pathways of BM 123 and BM 130 are shown in Figure 1. Compound III, N-[4-(2-hydroxyethylmethylamino)-2-butynyl]-2-pyrrolidone, was prepared as described by Sterk et al. (40) and purified by vacuum distillation; b.p. 169 °C (0.015 mm Hg). BM 123, N-[4-(2-chloroethylmethylamino)-2-butynyl]-2-pyrrolidone hydrochloride, was obtained from Compound III as previously described (40) in 48% yield. After recrystallization from an ethanol-ether mixture it had m.p. 140-142 °C in agreement with the reported m.p. of 142 °C (40).

Compound IV, N-[4-(2-hydroxymethylpyrrolidino)-2-butynyl]-2-pyrrolidone, was prepared through the Mannich reaction from N-(2-propynyl)-2-pyrrolidone, 2-hydroxymethylpyrrolidine, paraformaldehyde and a catalytic amount of cuprous chloride according to a method previously described (41); b.p. 174 °C (0.8 mm Hg), yield 54%. Anal. Calculated for $C_{13}H_{20}N_2O_2$: C 66.07; H 8.53; N 11.85. Found C 65.82; H 8.40; N 11.76.

BM 130, N-[4-(2-chloromethylpyrrolidino)-2-butynyl]-2-pyrrolidone hydrochloride was prepared by adding a solution of Compound IV (0.009 mol) in anhydrous chloroform (50 ml) to a solution of thionyl chloride (0.025 mol) in anhydrous chloroform (20 ml). The reaction mixture was stirred at room temperature for 2 hr and was then refluxed for 2 hr. After cooling, the solvent and the excess of thionyl chloride were removed under reduced pressure. The residue, a viscous dark oil, was dissolved in ethanol and boiled with activated charcoal. After filtration on celite, the solution was concentrated in vacuum, some ether added and the crystalline product recrystallized from an ethanol-ether mixture; m.p. 122-123 °C, yield 53%. Anal. Calculated for $C_{13}H_{19}N_2ClO \cdot HCl$: C 53.62; H 6.92; Cl 24.35; N 9.62. Found: C 53.35; H 7.20; Cl 24.10; N 9.30.

Compound V, N-[4-(3-hydroxypiperidino)-2-butynyl]-2-pyrrolidone, was synthesized by the Mannich reaction from N-(2-propynyl)-2-pyrrolidone, 3-hydroxypiperidine and paraformaldehyde as previously described (17); b.p. 179 °C (0.05 mm Hg), yield 60%.

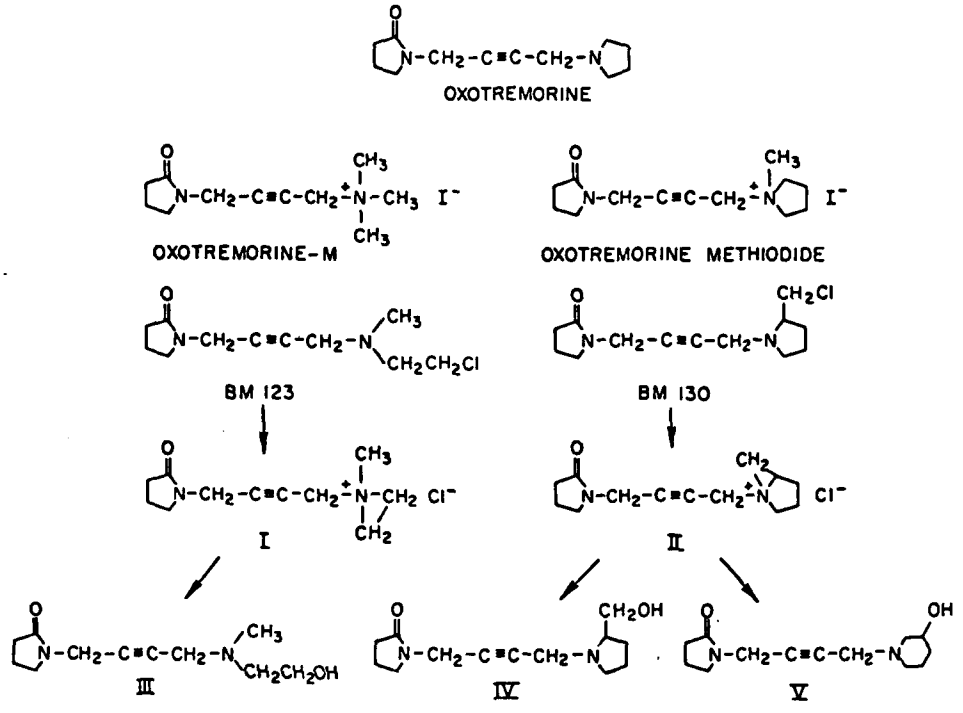

Fig. 1 Chemical structures of some oxotremorine analogues

BM 123 and BM 130 are transformed in aqueous solution at neutral pH to the aziridinium ions I and II, respectively. These are then hydrolysed to the aminoalcohols III-V.

4.1.2 Methods

(1) Formation and decomposition of aziridinium ions

The method used to quantitate aziridinium ions was based on that of Gill and Rang (42). A 2.0 mM solution of BM 123 or BM 130 in 30 mM sodium-potassium phosphate buffer (pH 7.0) was kept at constant temperature (23 or 37 °C) and aliquots (5 ml) were removed at various times. The cyclization was stopped by the addition of 0.2 N acetic acid (1 ml). Then 0.5 ml of 0.05 N sodium thiosulfate was added. After 20 min, residual thiosulfate was estimated by titration with standardized potassium triiodide (0.01 N). A starch indicator was used to determine the end point.

Chloride ion released during the cyclization was measured by argentometric titration according to the method of Kolthoff and Stenger (43).

Rate constants were estimated by fitting kinetic models to the data by an unweighted Gauss-Newton nonlinear regression routine programmed on a Hewlett Packard Model 67 calculator. Chloride release data were fitted to the equation $\lambda = 1 - e^{-k_1 t}$, where λ is the molar proportion of chloride released, k_1 is the apparent first order rate constant for the cyclization reaction and t is time. The concentration of aziridinium ions as a function of time was fitted to the equation

$$\emptyset = \frac{k_1}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t})$$

where $\emptyset$ is the molar ratio of aziridinium ion concentration to starting concentration of the parent compound, and k_1 and k_2 are the rate constants for the formation and decay of the aziridinium ion, respectively. Apparent rate constants (k_{app}) for chloride release from BM 130 were estimated over the pH range 5.2 - 7.6 by fitting the equation $\lambda = 1 - e^{-k_{app} t}$ to data (λ,t) over thime range 0 - 90 min. The resulting estimates of k_{app} at each pH were then used as input for a regression analysis according to the equation

$$k_{app} = \frac{k_1}{1 + 10^{(pK_a - pH)}}$$

where k_1 is the rate constant for chloride release from the free base of BM 130 and K_a is the acid dissociation constant of BM 130. This analysis provides estimates of pK_a and k_1.

(2) Isolated guinea pig ileum

A standard guinea pig ileum preparation was set up at 37 °C in Tyrode's solution (NaCl 137 mM, $NaHCO_3$ 12 mM, glucose 5 mM, KCl 2.7 mM, $MgSO_4$ 1 mM, NaH_2PO_4 0.4 mM and $CaCl_2$ 1.8 mM; pH 7.4) as previously described (44).

The muscarinic activity of BM 123 and BM 130 at different times after their dissolution (as 2 mM solutions) in 30 mM sodium-potassium phosphate buffer (pH 7.0) of room temperature was estimated by recording cumulative dose-response curves at intervals over a period of 12 hr. Three different preparations were used for each of these experiments. Potencies at each time point were expressed relative to that of oxotremorine-M used as a standard agonist. At the time of maximal muscarinic activity, dose-response curves to BM 123 and BM 130 were also obtained in the presence of methylatropine (20 nM) and hexamethonium (0.3 mM).

Separate experiments were performed to examine the effects of prolonged exposure of the ileum to BM 123, BM 130 and oxotremorine-M. For these experiments, BM 123 and BM 130 were cyclized as 2 mM solutions in 50 mM sodium-potassium phosphate buffer (pH 7.4) at room temperature for 60 and 15 min, respectively, when the concentration of aziridinium ions was near maximal. Control responses to oxotremorine-M, oxotremorine and BM 5 (see below under Drugs) were obtained. The tissue was then exposed to BM 123 (2 μM or 20 μM) or BM 130 (5 μM or 20 μM) for 30 min. All concentrations of BM 123 and BM 130 quoted in this study are those of the parent mustards. After the exposure, the tissue was washed with Tyrode's solution for 60 or 90 min. Sodium thiosulfate (0.1 mM) was always included in the Tyrode's solution during the first 15 min of the wash period in order to destroy any remaining aziridinium ions. Dose-response curves to oxotremorine-M were then obtained at intervals for the next 2.5 to 3.5 hr. Percent receptor occupancy (p) by BM 123 or BM 130 was calculated from the ratio of equiactive concentrations of oxotremorine-M at different times after the wash period and before the 30 min exposure (dose ratio) using the relationship 1 (45)

$$p = \frac{(\text{Dose ratio} - 1) \times 100}{\text{Dose ratio}} \qquad (1)$$

Tests for recovery of responses to oxotremorine and BM 5 were performed 2 hr after the exposure to BM 123 or BM 130. Receptor protection experiments were carried out with methylatropine (20 nM) which was allowed to equilibrate with the tissue for 30 min before the addition of BM 123 or BM 130. In control experiments, the ileum was exposed to oxotremorine-M (2 μM and 20 μM) for 30 min. Tests for recovery from desensitization and for subsequent changes in sensitivity of the ileum as a function of time were performed at 15 min intervals for 4.5 hr using oxotremorine-M.

Antagonists (Compounds IV and V) were tested against carbachol and were allowed to equilibrate with the preparation for 10 min. Tests for competitive antagonism were performed according to the procedure of Arunlakshana and Schild (46).

(3) Frog rectus abdominis

A standard frog rectus abdominis (Rana pipiens) preparation (47) was set up at 20 °C in aerated Clark-Ringer solution (NaCl 110 mM, $NaHCO_3$ 2.4 mM, glucose 10 mM, KCl 1.89 mM, NaH_2PO_4 0.08 mM and $CaCl_2$ 1.08 mM; pH 7.4). The tissue was allowed to rest for 1 hr before being stimulated with carbachol until constant responses were obtained. The preparation was exposed to each drug concentration for 5 min. Responses were expressed as a percentage of the maximal response to carbachol. BM 130 was cyclized for 15 min in a 2 mM solution in 50 mM sodium-potassium phosphate buffer (pH 7.4). BM 123 was cyclized for 1 hr as a 20 mM solution in 100 mM sodium-potassium phosphate buffer (pH 7.4) because of the high concentrations required to record dose-response curves in this preparation.

(4) Muscarinic receptor binding assays

Muscarinic receptor binding assays were run on segments of the guinea pig ileum that had been exposed to BM 123 and BM 130 for 30 min so that a comparison could be made between the estimates of receptor alkylation determined by [^{3}H]ligand binding methods and by application of equation 1 as described above. For these experiments, solutions (2 mM) of BM 123 and BM 130 in 50 mM sodium-potassium phosphate buffer (pH 7.4) were incubated at room temperature for 60 and 15 min, respectively, to allow formation of the aziridinium ions. The solutions were put on ice and used immediately. Segments (2.5 - 3.5 cm) of the guinea pig ileum were preincubated in Tyrode's solution at 37 °C gassed with 95% O_2 and 5% CO_2 for 15 - 30 min. An aliquot of BM 123 or BM 130 was added, and the tissue was incubated for an additional 30 min. The incubation was stopped by transferring the ileum to fresh Tyrode's solution containing 1.0 mM thiosulfate. The tissue was washed for 60 min, during which time, it was transferred to fresh Tyrode's solution every 20 min. Next, the ileum was mounted on a 1 ml pipet and the outer longitudinal muscle layer was gently rubbed off with a cotton swab. The longitudinal muscle was then minced with scissors and homogenized with a Potter Elvehjem glass homogenizer with teflon pestle in 50 mM sodium-potassium phosphate buffer (pH 7.4) to a concentration representing 10 mg original wet tissue weight/ml phosphate buffer. The tissue was rehomogenized with the Polytron (Brinkman) at setting #5 for 20 sec and filtered through three layers of cheese cloth. The homogenates were washed twice by centrifugation at 27,000 x g for 10 min followed by resuspension in fresh 50 mM sodium-potassium phosphate buffer (pH 7.4) with the final homogenate concentration being 4.0 mg original wet tissue weight/ml phosphate buffer. This homogenate was assayed subsequently for the binding of [^{3}H](-)3-quinuclidinyl benzilate ([^{3}H](-)QNB).

In another series of experiments, homogenates of the longitudinal muscle of the guinea pig ileum were incubated with BM 123 and BM 130 to determine what effect this would have on the binding of the specific muscarinic antagonist, [^{3}H]N-methylscopolamine ([^{3}H]NMS). For these experiments, solutions (2 mM) of BM 123 and BM 130 were incubated at 37 °C for 40 and 5 min, respectively, to allow formation of the aziridinium ions. The longitudinal muscle was removed from the guinea pig ileum and homogenized in 50 mM phosphate buffer to a concentration of 25 mg original wet tissue weight /ml phosphate buffer as described above. Aliquots (2 ml) of the homogenate were added to small tubes and preincubated at 37 °C for 10 min in a shaking water bath. Next, BM 123 or BM 130 was added to each tube at a final concentration of 10 μM, and the tubes were incubated for an additional 20 min. The reaction was stopped immediately by addition of atropine (0.1 mM) and thiosulfate (1.0 mM). The homogenates were washed 5 times by centrifugation at 27,000 x g for 10 min followed by resuspension in 3 ml of phosphate buffered saline (PBS; 181 mM Na^+, 100 mM Cl^-, 9.5 mM K^+, 50 mM PO_4, pH 7.4). After the first centrifugation, the homogenates were resuspended in PBS containing thiosulfate (1.0 mM). After the second, third, and fourth centrifugations, the homogenates were incubated for 5, 10, and 20 min, respectively, to allow time for the dissociation of atropine that might have been bound tightly to muscarinic receptors. After the fifth centrifugation, the pellets were frozen immediately at -20 °C and thawed the next day for assay of [^{3}H]NMS binding. The pellets were resuspended with a Potter Elvehjem homogenizer to a concentration representing 1.25 mg original wet tissue weight/ml 50 mM sodium-potassium phosphate buffer.

The specific binding of [^{3}H](-)QNB was measured according to the rapid filtration method of Yamamura and Snyder (48), and the binding of [^{3}H]NMS was measured by the centrifugation method described by Birdsall et al. (49). The following modifications of the assays were made. Aliquots (0.5 ml) of ileal homogenate were incubated with [^{3}H]ligand in a final volume of 2 ml containing 50 mM sodium-potassium phosphate buffer (pH 7.4). The incubation of tissue with [^{3}H](-)QNB lasted 60 min at 37 °C, and that for [^{3}H]NMS lasted 30 min at 30 °C. The incubation with [^{3}H]NMS was terminated by centrifugation at 27,000 x g for 10 min. Nonspecific binding for both [^{3}H]ligands was defined as the amount of radioactivity measured in the presence of 10 μM atropine. The binding data for [^{3}H]NMS were calculated assuming that both enantiomers of racemic [^{3}H]NMS bound nonspecifically to the same extent but that only the (-)enantiomer contributed to specific binding. Protein was measured by the Lowry method (50) using bovine serum albumin as the standard.

(5) Drugs

Oxotremorine sesquioxalate (51), oxotremorine-M (52), oxotremorine methiodide (53), and BM 5, N-methyl-N-(4-pyrrolidino -1-methyl-2-butynyl)-acetamide oxalate (54) were prepared as previously described. Other drugs and their sources were the

following: carbamylcholine chloride (Aldrich Chemical Co., Milwaukee, Wisc.), hexamethonium chloride and methylatropine bromide (K & K Laboratories, Plainview, NY), [^{3}H](-)QNB (40.2 Ci/mmole) and [^{3}H]NMS (84.8 Ci/mmole) (New England Nuclear Corporation, Boston, Mass.).

4.1.3 Formation and decomposition of aziridinium ions

The cyclization of BM 123 and BM 130 in phosphate buffer (pH 7.0) was monitored by measurement of chloride ion release and by utilizing the quantitative reaction of the formed aziridinium ions with thiosulfate. The latter reaction was also used to follow the hydrolysis of the aziridinium ions. Figure 2 shows the time course for the liberation of chloride ion and for the formation and decay of the aziridinium ion from BM 123 at 37 °C. The rate constant for the cyclization of BM 123, as measured by chloride release, was 1.14 hr^{-1} at 37 °C and 0.16 hr^{-1} at 23 °C. The rate constants for the decay of the aziridinium ion I were 1.98 and 0.56 hr^{-1}, respectively, at 37 °C and 23 °C. The aziridinium ion concentration reached its maximum after 40 min at 37 °C (28%) and after 150 min at 23 °C (18%).

Figure 3 illustrates the time course of chloride ion release and of aziridinium ion formation from BM 130 at 37 °C. The rate constant for the cyclization of BM 130, as measured from chloride ion release, was 63.6 hr^{-1} at 37 °C and 10.9 hr^{-1} at 23 °C. The time course of the decay at 37 °C of the aziridinium ion derived from BM 130 also is shown in Fig. 3. Rate constants of 0.75 and 0.18 hr^{-1}, respectively, were obtained at 37 °C and 23 °C. The aziridinium ion concentration reached its maximum after 5 min at 37 °C (84%) and after 20 min at 23 °C (77%).

We also studied the effect of pH on the rate of cyclization of BM 130 at 23 °C (Fig. 4). The rate of chloride release increased with pH up to pH 7.0 but did not increase much beyond this. The apparent rate constant for chloride release was in excellent agreement with the theoretical equation derived from the assumption that only the free base cyclizes under these conditions. Regression analysis yielded an estimated pK_a value of 6.37 and a first order rate constant for the free base of 14.2 hr^{-1}.

4.1.4 Identification of the hydrolysis products of BM 130

The hydrolysis of the aziridinium ion II may give rise to two isomeric amino-alcohols IV and V (Fig. 1). In order to determine the relative amounts of these, BM 130 (25 mg) was dissolved in 5 ml of 50 mM sodium-potassium phosphate buffer (pH 7.4) and the solution was left at room temperature for 48 hr. The solution was then made faintly alkaline by the addition of 1 N NaOH and extracted with dichloromethane (2 x 10 ml). The organic phase was dried over K_2CO_3. To 1 ml of the dichloromethane solution was added 0.1 ml of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) reagent (Regis Chemical Co., Morton Grove, Ill.) and the solution was heated in a closed tube at

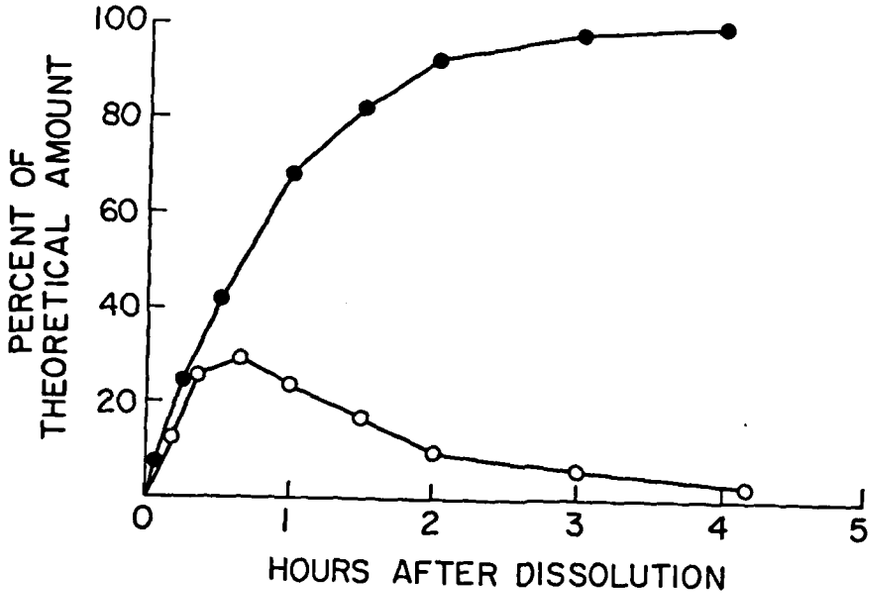

Fig. 2 Chloride release (●) and aziridinium ion formation and decay (○) from BM 123 at pH 7.0 and 37 °C

The ordinate shows concentrations of the ions as percentages of the maximum of 1 Eq/mole of BM 123. Ion concentrations were determined as described in Materials and Methods and are mean values of 3 separate experiments. The standard errors of the concentrations were always less than 2 percentage units.

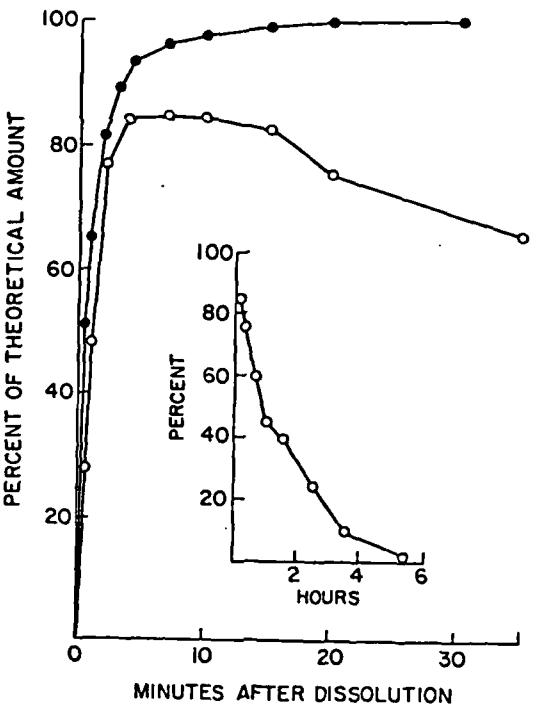

Fig. 3 Chloride release (●) and aziridinium ion formation and decay (○) from BM 130 at pH 7.0 and 37 °C

For details see Fig. 2.

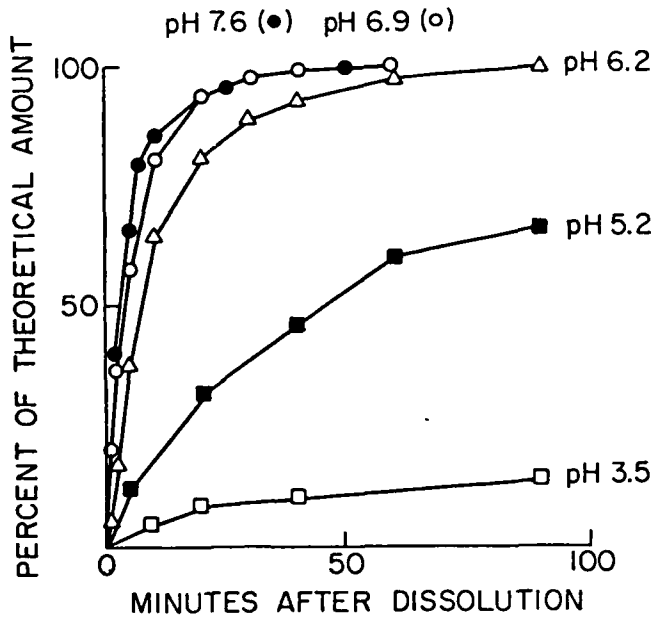

Fig. 4 pH Dependency of chloride release from BM 130

Chloride release was measured in phosphate buffer (pH 5.2 - 7.6) and in water (initial pH 3.5) at 23 °C as described in Materials and Methods. Data are from single experiments (pH 3.5 and 5.2) or from 2 separate experiments (pH 6.2, 6.9 and 7.6).

60 °C for 1 hr. A volume of 1 µl of this solution was injected to a Hewlett Packard 5840A Gas Chromatograph equipped with a flame ionization detector. A silanized glass column (2 m x 2 mm i.d.) containing 100/120 mesh Supelcoport coated with 5% QF-1 (Supelco Inc., Bellefonte, Penns.) was used. The column temperature was maintained at 200 °C. Injection port and detector temperatures were 225 °C. Two sharp peaks were observed (retention times 7.51 and 8.78 min) with relative areas of 5:3. Samples of Compounds IV and V derivatized as above gave single peaks and retention times of 7.50 and 8.75 min, respectively. A sample containing equimolar amounts of Compounds IV and V after derivatization gave two peaks with retention times of 7.50 and 8.75 min and relative areas 1:1. Therefore, both amino-alcohols are formed from BM 130 but Compound IV is the more abundant product. In addition to hydrolysis, aziridinium ions in phosphate buffer also are subject to nucleophilic attack by phosphate anion. Thus a phosphate adduct has been shown to be a decomposition product of propyl- benzilylcholine mustard in phosphate buffer (53). The extent of formation of such an adduct from BM 123 and BM 130 is not known.

4.1.5 Muscle contractile response: muscarinic

BM 123 and BM 130 were potent stimulants of the guinea pig ileum. The contractile responses elicited by the two compounds resembled those of oxotremorine-M and oxotremorine methiodide (Fig. 5). The muscarinic activity of solutions of BM 123 and BM 130 in phosphate buffer (pH 7.0) varied greatly with time after dissolution. For BM 123, maximal muscarinic activity was observed with a solution kept at room temperature for 2-4 hr, whereas solutions of BM 130 showed maximal activity 15-60 min after dissolution (Fig. 6). The short exposure (about 1.5 min) of the ileum to the low concentrations of BM 123 and BM 130 required to record dose-response curves did not alter the muscle responses to oxotremorine-M, i.e. no alkylation of the receptors was detected under these conditions. Therefore, BM 123 and BM 130 were bioassayed against oxotremorine-M. Relative potencies of BM 123 and BM 130 were obtained at the time of peak activity. The results are summarized in Table 1 and Fig. 5 which also include oxotremorine methiodide and carbachol. Methylatropine (20 nM) increased the ED_{50} values of BM 123 and BM 130 28-30 fold, whereas hexamethonium (0.3 mM) was without effect.

The muscarinic activity of solutions of BM 123 and BM 130 in phosphate buffer (pH 7.0) over a time period of 12 hr closely paralleled the aziridinium ion concentration as determined by thiosulfate consumption (Fig. 6). Forty-eight hours after dissolution, no aziridinium ion was detected in solutions of BM 123 and BM 130. At this time, solutions of BM 123 still showed weak muscarinic activity (less than 1% of the maximal activity), whereas solutions of BM 130 did not exhibit such activity. Treatment of a solution of BM 123 which had been kept at room temperature and pH 7.0 for 3 hr, and which therefore had maximal muscarinic activity (Fig. 6), with sodium thiosulfate

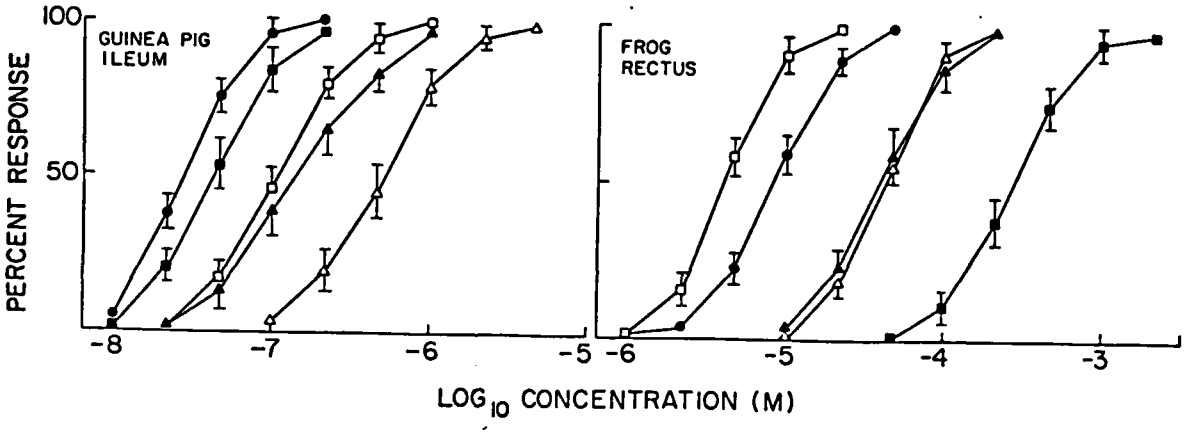

Fig. 5 Dose-response curves in the isolated guinea pig ileum and in the frog rectus abdominis muscle of oxotremorine-M (●), BM 123 (■), carbachol (□), BM 130 (▲) and oxotremorine methiodide (△)

Responses are expressed as a percentage of the maximum contraction elicited by oxotremorine-M (guinea pig ileum) or carbachol (frog rectus). Vertical bars show standard error.

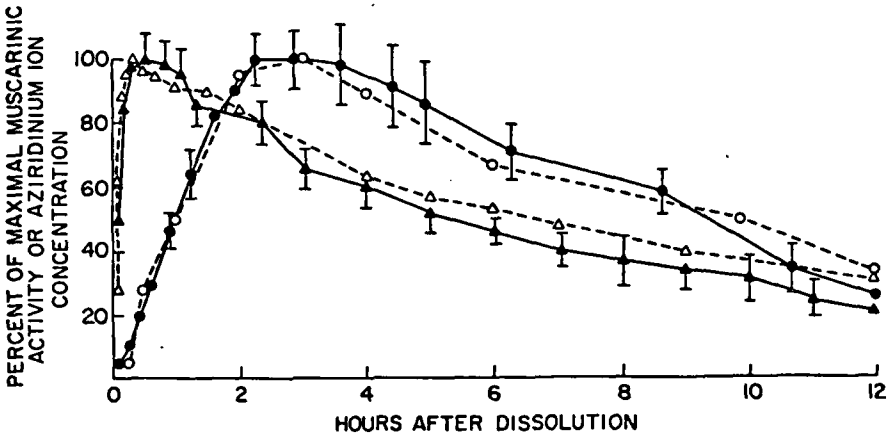

Fig. 6 Correlation of muscarinic activity and aziridinium ion concentration in solutions of BM 123 and BM 130

The solutions were kept in phosphate buffer at pH 7.0 and 23 °C. The muscarinic activity (●) and the aziridinium ion concentration (○) in the solution of BM 123 are expressed in terms of the muscarinic activity and the aziridinium ion concentration of a sample taken at 3 hr. For the solution of BM 130, the muscarinic activity (▲) and the aziridinium ion concentration (△) are given relative to those of a sample withdrawn at 20 min. Vertical bars show standard error of 3-4 experiments.

TABLE 1

Muscarinic and nicotinic activity of some oxotremorine analogues

The muscarinic and nicotinic activities are expressed as equipotent molar ratios (EPMR) ± standard error relative to oxotremorine-M. The number of preparations used is given in parenthesis.

Compound	Isolated guinea pig ileum EPMR	Frog rectus abdominis EPMR	$\frac{ED_{50}\ \text{Frog rectus}}{ED_{50}\ \text{Guinea pig ileum}}$
BM 123	1.4 ± 0.12 (6)	30.2 ± 2.4 (4)	6590
BM 130	5.6 ± 0.58 (6)	4.9 ± 0.79 (4)	270
Oxotremorine Methiodide	19.1 ± 2.0 (4)	5.0 ± 0.42 (4)	81
Carbachol	3.9 ± 0.30 (5)	0.48 ± 0.04 (4)	36
Oxotremorine-M	1.0	1.0	288

(0.1 mM for 15 min) almost completely abolished the muscarinic response. Since this solution had liberated only 40% of the theoretical amount of chloride (data not shown), it still contained about 60% of the original concentration of the parent mustard. Thiosulfate treatment of a solution of BM 130, which had been kept at room temperature for 30 min, totally abolished the muscarinic response. This solution had liberated virtually all the chloride (Fig. 4) and therefore did not contain the parent mustard. On the other hand, sodium thiosulfate (10 μM) added to the organ bath did not protect against the muscarinic actions of BM 123 and BM 130. Finally, thiosulfate treatment had no effect on the muscarinic activity of a solution of oxotremorine-M.

The alcohol (III) formed by hydrolysis of BM 123 was a muscarinic agonist which had less than 0.1% of the potency of oxotremorine-M. Both alcohols formed by hydrolysis of BM 130 were weak antagonists to carbachol on the isolated guinea pig ileum. Compound IV behaved like a competitive antagonist with a dissociation constant (K_B) of 11.9 ± 0.8 μM (S.E., n=4). Compound V appeared to be a non-competitive antagonist with a K_B value of 295 ± 60 μM (S.E., n=4).

To investigate possible alkylation of muscarinic receptors, segments of the isolated guinea pig ileum were exposed to BM 123 or BM 130 for 30 min. The length of the muscle was monitored continuously during this exposure. After the initial contraction, the length of the muscle increased steadily and in some experiments returned to near resting length before the end of the 30 min exposure period. In other experiments, the resting length was not restored during the 30 min exposure but was restored during the subsequent wash period. In some experiments, in which the muscle was exposed to the higher concentration (20 μM) of BM 123 and BM 130, there was a decrease in the resting length which was not influenced by washing or by atropine (1 μM). A similar phenomenon has been previously noted with acetylcholine mustard (55). The latter experiments were discarded.

Recovery from desensitization caused by exposure of the ileum to oxotremorine-M at 2 μM and 20 μM was complete within 50 and 80 min, respectively. Therefore, tests for recovery of responses to oxotremorine-M after exposure to BM 123 at 2 μM and BM 130 at 5 μM were begun after a wash period of 60 min. After exposure to a concentration of 20 μM of BM 123 and BM 130, the wash period was extended to 90 min. From the dose-response curves to oxotremorine-M before and at different times after the exposure of the ileum to BM 123 and BM 130, the percentage receptor occupation by BM 123 and BM 130 was calculated (Table 2). Corrections were made for the small changes in the sensitivity of the ileum to oxotremorine-M occurring with time as evidenced in the control experiments. BM 123 and BM 130 caused a dose-dependent decrease of the sensitivity of the guinea pig ileum to oxotremorine-M. No significant recovery of responses to oxotremorine-M was observed over a time period of 4.5 hours after the exposure.

TABLE 2

Percent occupancy of muscarinic receptors in the isolated guinea pig ileum by BM 123 and BM 130

The ileum was exposed to each concentration of BM 123 and BM 130 for 30 min. In the protection experiments, methylatropine (20 nM) was allowed to equilibrate with the preparation for 30 min before the addition of BM 123 or BM 130. Each value represents the mean ± standard error. The number of animals used is given in parenthesis.

Tissue treatment	Percent receptor occupancy						Percent decrease of response to BM 5[b]
	Inhibition of contractile responses					Inhibition of [^{3}H]QNB binding[a]	
	Hours after exposure						
	1.0	1.5	2.5	3.5	4.5		
BM 123 20 µM (4)		94.0± 3.1	94.0±2.9	92.8±2.8	92.3±2.1	81.9±1.8	100
BM 123 2 µM (5)	49.4±6.9	48.0± 8.0	49.4±6.4	50.6±4.9		61.0±2.7	63±8
Methylatropine and BM 123 20 µM (3)		32.3± 5.8	36.4±2.6	39.6±2.1	39.3±1.8		61±5
Methylatropine and BM 123 2 µM (3)	11.7±2.7	8.9± 4.7	4.9±4.9				17±6
BM 130 20 µM (4)		85.4± 2.8	85.6±2.9	85.8±2.6	86.2±2.7	79.8±3.0	100
BM 130 5 µM (3)	64.5±2.0	62.9± 3.5	61.3±6.4	62.1±5.9		66.5±2.5	69±7
Methylatropine and BM 130 20 µM (3)		38.6±10.9	38.3±8.5	39.4±6.7	41.8±6.5		66±6
Methylatropine and BM 130 5 µM (3)	27.9±1.8	25.3± 5.5	23.9±3.5				26±3

[a] Percent inhibition of specific [^{3}H](-)QNB binding as compared to untreated tissue.
[b] Percent decrease of the height of the contractile response as compared to the height of the response before incubation with BM 123 or BM 130.

A test of the specificity of action of BM 123 and BM 130 was made by measuring the protecting action of methylatropine against the irreversible effects. Methylatropine (20 nM) significantly reduced the fraction of receptors occupied by BM 123 at 20 μM and by BM 130 at 20 μM and 5 μM and almost fully protected against the irreversible actions of BM 123 at 2 μM (Table 2).

We also studied changes in the height of the contractile responses of oxotremorine-M, oxotremorine and BM 5, a partial muscarinic agonist (54), caused by the treatments of the ileum with BM 123 and BM 130 described above. In this way, we hoped to obtain an independent verification of the method used to determine the extent of receptor alkylation. The maximal response to oxotremorine-M was not depressed in any of the experiments summarized in Table 2. The response to oxotremorine was always depressed after exposure of the ileum to BM 123 at 20 μM and in two of the four expriments in which the ileum was exposed to BM 130 at 20 μM. In all other experiments, the maximal response to oxotremorine was virtually unaffected. Finally, the response to BM 5 was abolished in two experiments and depressed in all others although the depression was small in those experiments in which methylatropine protected against extensive receptor alkylation (Table 2).

4.1.6 Muscle contractile response: nicotinic

BM 123 and BM 130 as well as oxotremorine-M and oxotremorine methiodide caused slow contractions of the frog rectus abdominis muscle similar to those elicited by carbachol (Fig. 5). The equipotent molar ratios relative to oxotremorine-M are summarized in Table 1, which also shows the ratio of the ED_{50} values for contraction of the frog rectus and of the guinea pig ileum for each compound. This ratio was calculated to obtain a measure of the selectivity for muscarinic receptors. There was no clear evidence of irreversible actions of BM 123 or BM 130 under the conditions used to record dose-response curves in the frog rectus abdominis preparation.

4.1.7 Muscarinic receptor binding activity: muscle

Treatment of the guinea pig ileum with BM 123 and BM 130 caused an irreversible inhibition of the binding of [^{3}H](-)NMS. Homogenates of the longitudinal muscle of the guinea pig ileum were incubated with BM 123 and BM 130 (10 μM) for 20 min and then washed extensively. Control homogenates were treated identically except for exposure to BM 123 and BM 130. Measurements of the specific binding of [^{3}H](-)NMS to the homogenates were made at racemic [^{3}H]ligand concentrations of 0.069, 0.17, 0.44, 1.1, and 2.8 nM, and the results were analyzed by nonlinear regression analysis to determine the effect of incubation with BM 123 and BM 130 on the dissociation constant and binding capacity of [^{3}H](-)NMS. The control binding isotherm was consistent with a Langmuir isotherm having a dissociation constant of 0.18 nM and binding capacity of 1.52 pmol/mg protein. Prior treatment of the

homogenates with BM 123 and BM 130 caused a significant reduction in the binding capacity to 0.656 and 0.851 pmol/mg protein, respectively, without significantly affecting the apparent affinity of [^{3}H](-)NMS. Nonlinear regression and analysis of variance showed no significant increase in residual error ($F = 0.905$, $p = 0.438$) when the data were fitted sharing the control estimate of the dissociation constant among the three binding isotherms. We have found a greater reduction in receptor capacity after an incubation of tissue with BM 123 or BM 130 when the reaction was not stopped immediately by addition of atropine.

Binding assays with [^{3}H](-)QNB were run on ilea which had been incubated with BM 123 or BM 130 for 30 min to determine if the extent of receptor alkylation determined pharmacologically was comparable to that estimated by measuring the reduction in [^{3}H](-)QNB binding. Segments of the guinea pig ileum were incubated with BM 123 (2 μM and 20 μM) and BM 130 (5 μM and 20 μM) for 30 min and then washed extensively as described in the Methods. Control ilea were treated the same way except for exposure to BM 123 and BM 130. [^{3}H](-)QNB binding was measured at a concentration of 0.4 nM in homogenates of the longitudinal muscle of the guinea pig ileum. The percentage of receptors alkylated by BM 123 and BM 130 was calculated as the percent inhibition of specific [^{3}H](-)QNB binding. Because experiments with [^{3}H]NMS showed that the inhibitory effect of BM 123 and BM 130 on muscarinic receptor binding properties was due to a selective reduction in binding capacity without an affect on affinity, it was assumed that the reduction in the binding of [^{3}H](-)QNB was proportional to the decline in receptor capacity. The results of these experiments are shown in Table 2. It can be seen that there is general agreement among the various estimates of receptor alkylation caused by treatment of the ileum with BM 123 and BM 130.

4.1.8 Effects of BM 123 on muscarinic receptor binding

Collectively, our earlier results are consistent with the postulate that BM 123 binds covalently to the recognition site of the muscarinic receptor. In another series of experiments we examined in detail the influence of BM 123 on the binding properties of muscarinic receptors in the rat cerebral cortex. In principle, the rate of alkylation of a receptor by a reactive ligand should be proportional to receptor occupancy. By measuring the rate of receptor alkylation over a range of ligand concentrations it should be possible to estimate both the rate constant for alkylation and the apparent affinity of the reactive ligand for the receptor. The results of such studies are presented below and are shown to be consistent with the postulate that BM 123 discriminates kinetically among different agonist subclasses of the muscarinic receptor in rat cerebral cortex. We have also estimated the affinity of the aziridinium ion by competitive inhibition of [^{3}H]ligand binding to the muscarinic receptor at 0°, conditions under which little or no receptor alkylation occurs, and have found general agreement among the

estimates of the dissociation constants determined kinetically and at equilibrium.

(1) Formation of the aziridinium ion

Unless indicated otherwise, solutions of BM 123 were first incubated in vitro to allow formation of the aziridinium ion before the solution was used in an experiment. Stock solutions of BM 123 were made up at a concentration of 2.0 mM in 0.05 M phosphate buffer (81 mM Na^+, 9.5 mM K^+, 50 mM PO_4) at pH 7.4 or at a higher concentration of 20 mM in 0.1 M phosphate buffer (188 mM Na^+, 5.8 mM K^+, 100 mM PO_4) at pH 7.8. These solutions were incubated immediately at 37° for 40 min, placed on ice, and used as soon as possible. This incubation procedure represents conditions under which the aziridinium ion reaches its peak concentration of 28% of the original parent mustard concentration. BM 123 and its alcoholic hydrolysis product were obtained as described previously.

(2) Tissue preparation

In most of the experiments described in this report, homogenates of rat cerebral cortex were incubated with BM 123 for a given length of time, washed extensively, and then assayed for [^{3}H]ligand binding to estimate the amount of receptors alkylated by BM 123. The tissue preparation and incubation conditions for these experiments were as follows. The freshly excised cerebral cortex from male Sprague Dawley rats (200 - 250 g) was homogenized in 0.05 M phosphate buffer at pH 7.4 with a Potter Elvehjem glass homogenizer and teflon pestle to a concentration of approximately 2.5% (w/v). The homogenate was centrifuged at 30,000 x g for 10 min and resuspended in 0.05 M phosphate buffer containing $MgSO_4$ (1.0 mM) to a concentration representing 25 mg original wet tissue weight per ml buffer. The homogenate was pipetted (2 ml aliquots) into small plastic tubes and preincubated at 37° for 10 min in a Dubnoff shaking water bath. An aliquot (100 µl) of a solution of BM 123 that had been incubated in vitro to allow formation of the aziridinium ion was added, and the tissue was incubated further at 37° for various times. The reaction was stopped immediately by the addition of atropine (0.1 mM) and sodium thiosulfate (1.0 mM). The tissue was washed five times to remove the atropine, thiosulfate, BM 123, and its transformation products. Washing was accomplished by centrifugation at 27,000 x g for 10 min followed by resuspension of the pellet in 3 ml of phosphate buffered saline (181 mM Na^+, 100 mM Cl^-, 9.5 mM K^+, 50 mM PO_4) at pH 7.4. After the first centrifugation, the pellets were resuspended in buffer containing thiosulfate (1.0 mM). After the second, third and fourth centrifugations, the homogenates were incubated at 37° for 5, 10 and 20 min, respectively, to allow sufficient time for the dissociation of atropine that might have been tightly bound to the muscarinic receptor. After the fifth centrifugation the cortical pellets were frozen immediately. On the next day, when [^{3}H]ligand binding was measured, the pellets were thawed and

resuspended to a concentration representing 10 mg original wet tissue weight/ml phosphate buffer. The washing procedure described above was sufficient to restore the binding of [^{3}H](-)QNB to control levels following incubation of cortical homogenates with atropine (0.1 mM) for 1 hr.

In other experiments, various nonlabelled drugs including BM 123 and its transformation products were included in the binding assay during the incubation of cortex with [^{3}H]ligand. For these experiments, rat cerebral cortex was homogenized as described above and centrifuged at 30,000 x g for 10 min. The pellet was resuspended in 0.05 M phosphate buffer to a concentration representing 10 mg original wet tissue weight/ml buffer.

(3) Binding assays

The binding of the specific muscarinic antagonist [^{3}H](-)QNB (33.4 Ci/mmole; New England Nuclear; Boston, Massachusetts) was measured using the rapid filtration method of Yamamura and Snyder (56) with the following modifications. Brain homogenate (0.1 ml) was incubated with [^{3}H](-)QNB in a final volume of 2 ml containing 0.05 M phosphate buffer, at pH 7.4, and 1.0 mM $MgSO_4$. The incubation was always carried out at 37° for 1 hr. Membrane bound [^{3}H](-)QNB was trapped subsequently by rapid vacuum filtration of the incubation mixture over glass fiber filters (Whatman, GF/B). The filters were rinsed with three aliquots (4 ml each) of ice cold saline. All assays were run in triplicate, and nonspecific binding was determined in the presence of 10 μM atropine. Protein was measured by the method of Lowry et al. (50) using bovine serum albumin as the standard. The binding of the specific muscarinic antagonist [^{3}H](-)NMS (84.8 Ci/mmole; New England Nuclear) was measured in the same manner as that described for [^{3}H](-)QNB except for the following modifications. Since equilibrium is achieved more rapidly with [^{3}H](-)NMS than with [^{3}H](-)QNB (6), the incubation of tissue with [^{3}H](-)NMS can be shortened and run at lower temperatures. In our experiments, incubations with [^{3}H](-)NMS were done at 30° for 30 min or 0° for 60 min. The incubations were carried out in small plastic scintillation vials (4 ml; Omnivials), and membrane bound radioactivity was trapped at the end of the incubation by centrifugation at 27,000 x g for 10 min. The pellets were washed superficially with two aliquots (3 ml each) of ice-cold saline, and the tubes were inverted and allowed to dry for one hour. The insides of the tubes were blotted dry without disturbing the pellet, and scintillation cocktail was added. The tubes were then vortexed immediately to resuspend the membranes in the scintillation fluid.

The procedure for measuring the binding of the specific muscarinic agonists [^{3}H](+)cis-2-methyl-4-dimethylaminomethyl-1,3-dioxolane methiodide ([^{3}H](+)CD; 38.1 Ci/mmole; New England Nuclear) and [^{3}H]oxo-M (82.5 Ci/mmole; New England Nuclear) was essentially the same as that described for [^{3}H](-)NMS except that

more tissue was used in the incubation (10 mg, based on original wet tissue weight). The incubations were run at 0° for one hour.

(4) Kinetic theory

The kinetics of the alkylation of the muscarinic receptor by BM 123A were investigated over a range of BM 123 concentrations so that it would be possible to determine both the affinity of the aziridinium ion for the receptor and its rate constant for alkylation. The kinetic parameters were estimated from the experimental data by nonlinear least squares regression analysis according to the equations 5 and 6, derived below.

The simplest model to describe the interaction of BM 123A (A) with the muscarinic receptor (R) is:

$$A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AR \xrightarrow{k_2} A\text{-}R \qquad (1)$$

where k_{+1} and k_{-1} are the rate constants for association and dissociation, respectively, of the reversible BM 123A receptor complex (AR), and k_2 is the rate constant for formation of the alkylated receptor complex (A-R). If the values of k_{+1} and k_{-1} are large with respect to k_2, then the rate of loss of unalkylated receptors can be described by the following differential equation:

$$\frac{dR_f}{dt} = \frac{-k_2 \, A \, R_f}{A + K_A} \qquad (2)$$

In this equation, R_f is equal to the unalkylated receptors (R_f = R + AR) and K_A is equal to the apparent dissociation constant of the AR receptor complex ($K_A = k_{-1}/k_{+1}$). During an incubation of BM 123 with muscarinic receptors at 37°, the concentration of A does not remain constant. In a previous study of the formation and hydrolysis of BM 123A in phosphate buffer at neutral pH, it was shown that the aziridinium ion reaches its peak concentration 40 min after dissolution in aqueous buffer at 37°, and that its concentration slowly declines to half of its peak concentration approximately 60 min later. Regression analysis of our data showed that the aziridinium ion decayed from its peak concentration of 28% in a manner well described by the simple exponential equation:

$$A = A_o e^{-k_o t} \qquad (3)$$

where A_o is the peak concentration of the aziridinium ion, k_o is the observed rate constant for decay of the aziridinium ion ($k_o = 0.0117 \text{ min}^{-1}$), and t denotes time after the aziridinium ion reaches its peak concentration. As described above, BM 123 was first incubated for 40 min at 37° to allow the aziridinium ion to reach its peak concentration before incubation with tissue homogenate so that the aziridinium ion concentration could be

predicted at various times during the incubation by the simple exponential equation (Eq. 3). Substituting equation 3 for A in equation 2 yields:

$$\frac{dR_f}{dt} = \frac{-k_2 A_o e^{-k_o t} R_f}{A_o e^{-k_o t} + K_A} \quad (4)$$

Integrating equation 4 and evaluating the integral over the time interval beginning with t = 0 yields:

$$Y = P\left(\frac{A_o e^{-k_o t} + K_A}{A_o + K_A}\right)^{k_2/k_o} \quad (5)$$

In this equation, Y is the percentage of remaining unalkylated receptors, and P is the estimate of 100% binding. The value of Y was estimated by measuring [^{3}H]ligand binding to cortical membranes which had been previously incubated with BM 123 for various times. The kinetics of the loss [^{3}H](-)QNB binding sites were adequately described by the two-site version of equation 5:

$$Y = P\left[a\left(\frac{A_o e^{-k_o t} + K_H}{A_o + K_H}\right)^{k_H/k_o} + (1 - a)\left(\frac{A_o e^{-k_o t} + K_L}{A_o + K_L}\right)^{k_L/k_o}\right] \quad (6)$$

where K_H and K_L are the dissociation constants and k_H and k_L are the rate constants of alkylation of the high and low affinity sites, respectively, and a is the proportion of high affinity sites.

(5) Analysis of equilibrium binding data

The equilibrium binding parameters of [^{3}H](-)NMS and nonlabelled drugs were determined by fitting the experimental data to the equations described below using nonlinear least squares regression analysis. The binding data for [^{3}H](-)NMS were calculated assuming that both enantiomers of racemic [^{3}H]NMS bound nonspecifically to the same extent but that only the (-)enantiomer contributed to specific binding. The dissociation constant (K_{NMS}) and receptor capacity (B_{max}) of [^{3}H](-)NMS were determined by regression analysis of the [^{3}H]ligand binding isotherm according to the following equation:

$$B = X\ B_{max}\ /(X + K_{NMS}) \quad (7)$$

where B is specifically bound [^{3}H](-)NMS, and X is the free concentration of [^{3}H](-)NMS. The binding parameters of nonlabelled ligands were determined by measuring their ability to inhibit [^{3}H]ligand binding competitively and by fitting the data to the one and two-site equations (Eq. 8, 9) shown below:

$$Y = P \frac{1}{1 + x/K'} \quad (8)$$

In this one-site equation, Y is the percent of [^{3}H]ligand bound, P is the estimate of 100% [^{3}H]ligand bound in the absence of the inhibitor, x is the concentration of nonlabelled inhibitor, and K' is the apparent dissociation constant of the nonlabelled inhibitor. In the following two-site equation

$$Y = P \left(\frac{a}{1 + x/K_H'} + \frac{(1-a)}{1 + x/K_L'} \right) \quad (9)$$

a is the proportion of high affinity sites and K_H' and K_L' are the apparent dissociation constants of the high and low affinity sites, respectively. The apparent dissociation constants (K') were corrected to give the true dissociation constants (K) using the following equation:

$$K = K'/(1 + y/K_y) \quad (10)$$

where y is the concentration of the [^{3}H]ligand and K_y is the dissociation constant of the [^{3}H]ligand, which was determined independently. The concentration of nonlabelled inhibitor required for half-maximal occupation of receptors (X_{50}) was calculated from the IC_{50} value (concentration of inhibitor that caused half-maximal inhibition of specific [^{3}H]ligand binding) according to the following equation:

$$X_{50} = IC_{50}/(1 + y/K_y) \quad (11)$$

As described below, low concentrations of [^{3}H]oxo-M and [^{3}H](+)CD were used to label selectively a subpopulation of agonist binding sites designated as the superhigh affinity site. The dissociation constants (K_Y) of oxo-M and (+)CD for the site were determined by running oxo-M/[^{3}H]oxo-M and CD/[^{3}H](+)CD competition experiments and correcting the respective IC_{50} values according to the following equation which is a special case of equation 10:

$$K_Y = IC_{50} - y \quad (12)$$

where y is the concentration of [^{3}H]oxo-M or [^{3}H](+)CD used in the experiment which was 1.0 nM or 2.0 nM, respectively.

(6) Alkylation of specific binding sites by BM 123

Treatment of the rat cerebral cortex with BM 123 caused an irreversible inhibition of the binding of [^{3}H](-)NMS. Homogenates of the rat cerebral cortex were incubated with BM 123 for 20 min and 1 hr and then washed extensively. Control homogenates were treated identically except for exposure to BM 123. Measurements of the specific binding of [^{3}H](-)NMS were made at various [^{3}H]ligand concentrations, and these results are

shown in Figure 7. Nonlinear regression analysis showed that the control binding measurements were consistent with a single binding site having a dissociation constant of 0.044 nM and a binding capacity of 0.68 pmole/mg protein. BM 123 treatment caused a selective reduction in the binding capacity of [^{3}H](-)NMS without significantly affecting the apparent affinity. Cortical homogenates that had been incubated with BM 123 (1.0 mM) for 20 and 60 min showed reduced binding capacities for [^{3}H](-)NMS of 0.16 and 0.078 pmole/mg protein, respectively. Analysis of variance showed no significant increase in residual error when the data were fitted by nonlinear regression analysis sharing the control estimate of the dissociation constant among the three binding isotherms ($F_{2,12} = 0.136$, $P = 0.87$).

Experiments were run to determine if a recovery of [^{3}H](-)NMS binding could be detected in homogenates that had been treated with BM 123 and then incubated at 37° for periods up to 4 hr. In these experiments, homogenates of rat cerebral cortex were incubated with BM 123 (10 μM) for 20 min at 37°. The reaction was stopped by a twenty-fold dilution with 1.0 mM thiosulfate in 0.05 M phosphate buffer. The homogenate was incubated an additional 20 min at 37° to inactivate the residual aziridinium ion and then washed twice by centrifugation at 30,000 x g for 10 min followed by resuspension in fresh phosphate buffer. The homogenate was then incubated at 37° for 4 hr. At the beginning and at various times (0.5, 1, 2, and 4 hr) during the incubation, aliquots of the homogenate were removed and assayed for [^{3}H](-)NMS binding at 0° using a [^{3}H]ligand concentration of 0.4 nM. In control homogenates, specific [^{3}H](-)NMS binding declined from an initial value of 0.574 $\pm$ 0.031 pmol/mg protein to 0.485 $\pm$ 0.017 pmol/mg protein after 4 hr at 37°. Specific [^{3}H](-)NMS binding in the BM 123 treated homogenate was initially 0.113 $\pm$ 0.008 pmol/mg protein and did not change significantly during the 4 hr incubation at 37°. When specific [^{3}H](-)NMS binding in the BM 123 treated homogenate is expressed as a percent of control, the binding values increase from an initial value of 20% to a value of 24% after incubation of the homogenate at 37° for 4 hr. Whether this small, apparent rate (1%/hr) of recovery of binding is due to a dissociation of the alkylated receptor complex or to proteolysis of receptors in the control homogenate is unclear from the present data.

Experiments were run to determine the effect of tissue concentration on the rate of muscarinic receptor alkylation by BM 123. Homogenates of the rat cerebral cortex were incubated with BM 123 (1 μM) for various times, washed extensively, and then assayed for [^{3}H](-)QNB binding. The results in Figure 8 show that the relative rate at which BM 123 caused an irreversible inhibition of [^{3}H](-)QNB binding, expressed as a percent of control, was independent of tissue concentration from 5 to 25 mg original wet tissue weight/ml. The half-time for the loss of [^{3}H](-)QNB binding was approximately 40 min in both cases. The remainder of the kinetic experiments were run using a final tissue concentration of 25 mg of original tissue (wet weight) per ml buffer as described in Materials and Methods. No

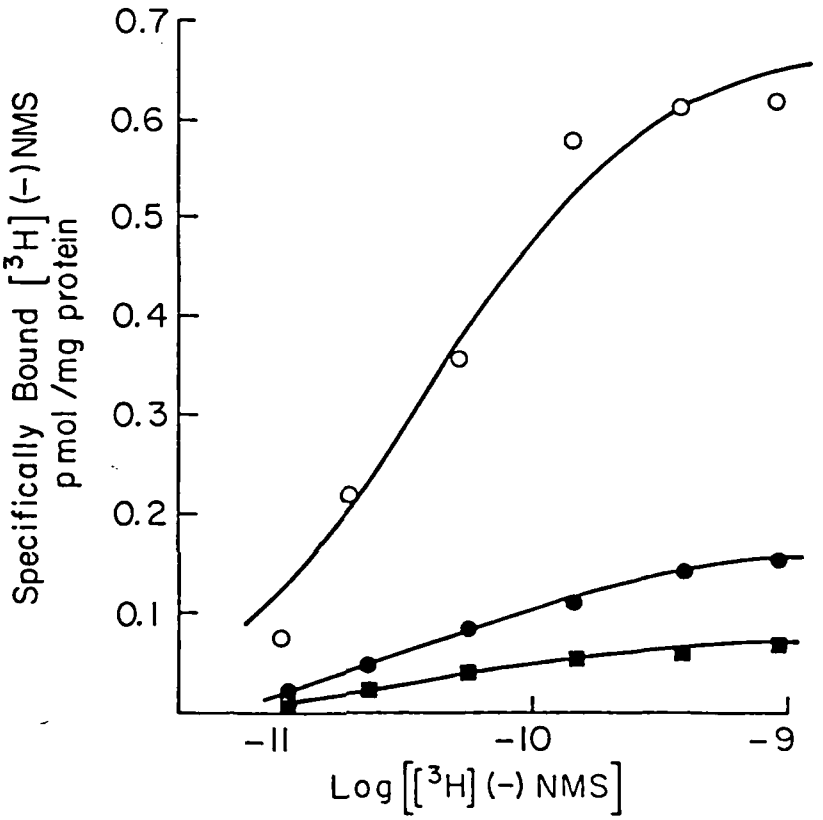

Fig. 7 The effect of BM 123 treatment on [^{3}H](-)NMS binding to homogenates of the rat cerebral cortex

Specific [^{3}H]NMS binding was measured at 30° in control homogenates (○) and homogenates which had been incubated at 37° with BM 123 (1.0 mM) for 20 min (●) and 60 min (■). The data points represent the mean binding values of five experiments, each done in triplicate. The curves represent the least squares fit to the data.

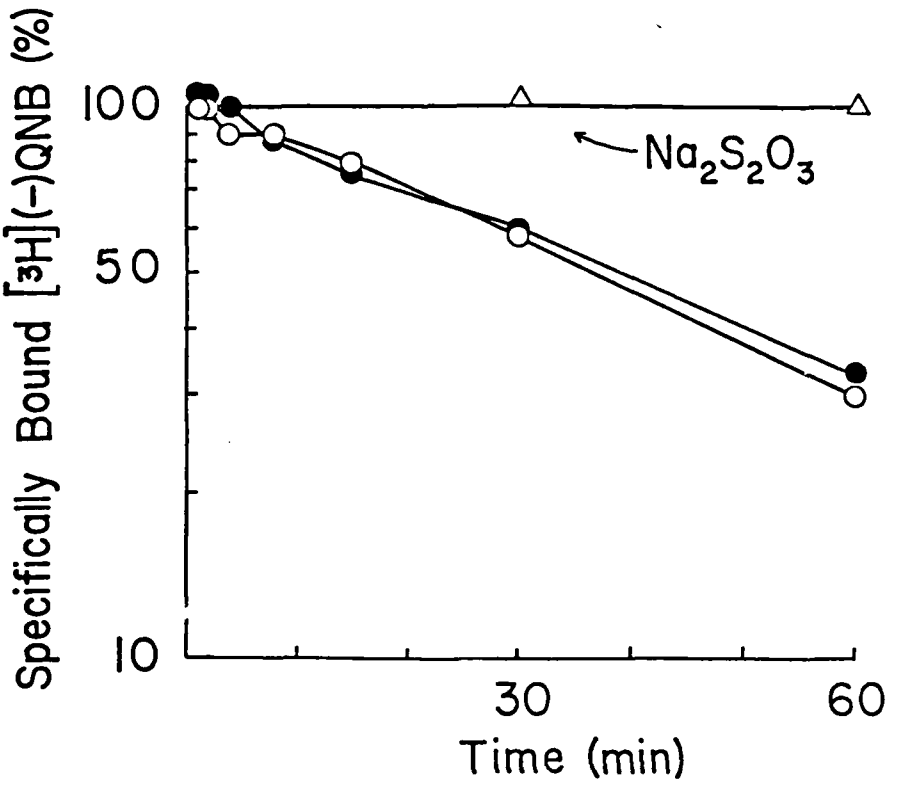

Fig. 8 The effect of tissue concentration and sodium thiosulfate (1.0 mM) on the rate of alkylation of [^{3}H](-)QNB binding sites by BM 123 (1.0 mM)

Homogenates of the rat cerebral cortex were incubated at 37° with BM 123 for the indicated times, washed extensively, and then assayed for specific [^{3}H](-)QNB binding at 37° using a [^{3}H]ligand concentration of 0.4 nM. The concentrations of the cortical homogenates were 5 mg (○) and 25 mg (●,△) original wet tissue weight/ml phosphate buffer. The data points represent the mean binding values of 3 experiments, each done in triplicate.

significant inhibition of [^{3}H](-)QNB binding was observed when the incubation of brain homogenate with BM 123 (1.0 μM) was carried out in the presence of sodium thiosulfate (1.0 mM) (see figure 3). Also, no significant inhibition of [^{3}H](-)QNB binding was detected in cortical homogenates which had been treated simultaneously with BM 123 (1.0 mM) and atropine (0.1 mM) and then incubated for 1 hr at 37° (data not shown). These results showed that the addition of atropine (0.1 mM) to an incubation of BM 123 with cortical homogenate immediately stopped the covalent reaction even when BM 123 was present at concentrations as high as 1.0 mM.

The rate at which BM 123 alkylated [^{3}H](-)QNB binding sites in cortical homogenates was sensitive to temperature. Reducing the incubation temperature from 37° to 25° caused a seven-fold increase in the half-time for the loss of [^{3}H](-)QNB binding sites when cortical homogenates were incubated with BM 123 at a concentration of 10 μM (data not shown). At 0°, no significant alkylation of [^{3}H](-)QNB binding sites occurred after incubating homogenates for 1 hr with BM 123 at concentrations from 1.0 - 100 μM. After incubating cortical homogenates with 1.0 mM BM 123 for 1 hr at 0°, a 10% loss in [^{3}H](-)QNB binding was measured.

(7) Affinity of BM 123 and its transformation products for binding sites

The slow rate at which BM 123 inactivated [^{3}H](-)QNB binding sites at low temperature (0°) enabled the affinity of BM 123 and its transformation products to be determined in competition experiments with [^{3}H]oxo-M and [^{3}H](-)NMS. Preliminary binding experiments with [^{3}H]oxo-M and [^{3}H](-)NMS on the rat cerebral cortex showed that equilibrium was achieved within 1 hr at 0° and that binding measurements after 1 - 3 hr of incubation were essentially equivalent. To assess the potency of the aziridinium ion, aliquots of a solution of BM 123 that had been incubated *in vitro* at 37° for 40 min to allow formation of the aziridinium ion were added directly into the competitive binding assay at 0°. The data were calculated assuming that the concentration of the aziridinium ion was equivalent to 28% of the parent mustard concentration. To determine the potency of the parent mustard, solutions of BM 123 were made up in dilute HCl at 0° to prevent formation of the aziridinium ion, and aliquots of these solutions were added to the competitive binding assay in the presence of 0.1 mM sodium thiosulfate. The addition of thiosulfate to the assay did not significantly affect the ligand/[^{3}H]oxo-M competition curves. It can be seen in Figure 9 that BM 123A and oxo-M have nearly equal affinity for the binding site labelled by [^{3}H]oxo-M with their respective IC_{50} values being 3.5 nM and 2.8 nM. In contrast, the parent 2-chloroethylamine (BM 123) and the alcoholic hydrolysis product were 100 - 200 fold less potent with IC_{50} values of 0.63 μM and 2.5 μM, respectively. Previous experiments by Birdsall et al. (57) have shown that at a concentration of 1.0 nM, [^{3}H]oxo-M labels almost exclusively a very high affinity agonist subpopulation of muscarinic receptors designated as the superhigh affinity site. The dissociation

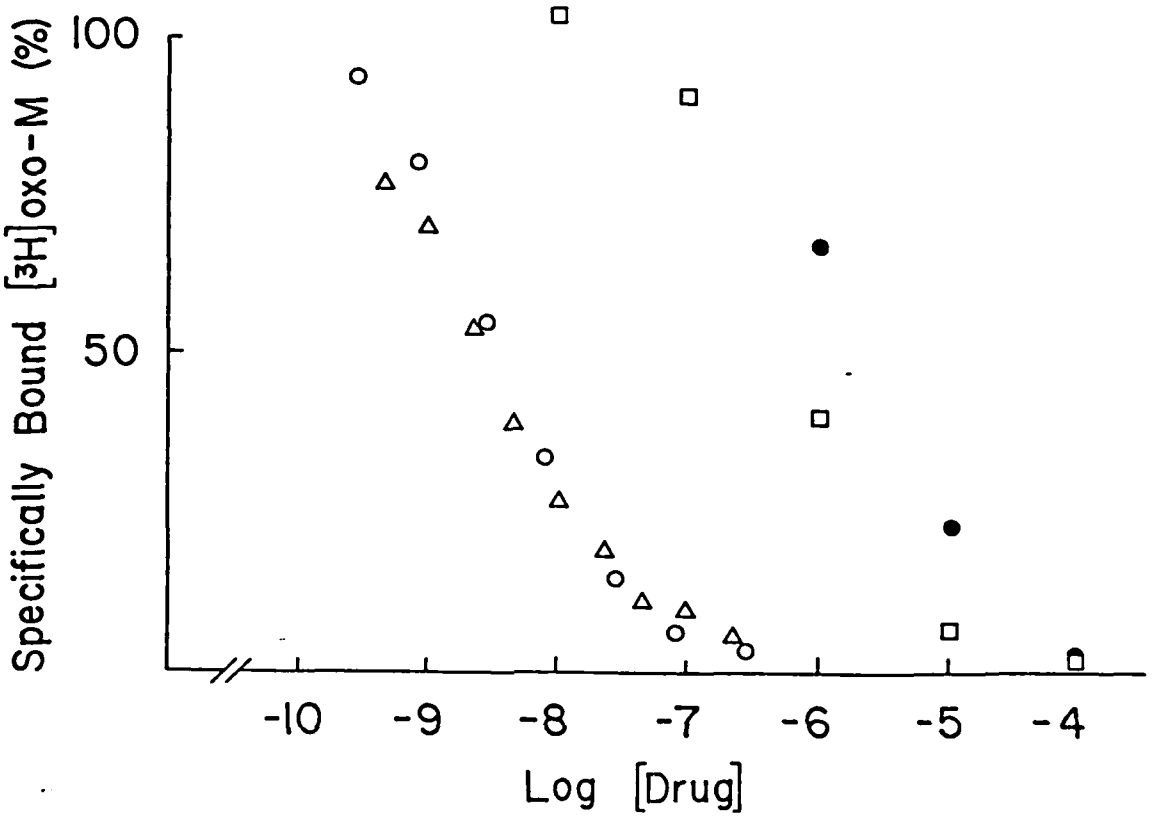

Fig. 9 The competitive inhibition of [^{3}H]oxo-M binding to the rat cerebral cortex by oxo-M (△), BM 123 (□), its aziridinium ion (○) and alcoholic hydrolysis product (●).

The specific binding of [^{3}H]oxo-M was measured at 0° in the presence of the indicated concentrations of the various compounds. The concentration of [^{3}H]oxo-M was 1.0 nM. Each data points represents the mean binding of 3 experiments, each done in triplicate.

constant (K_{SH}) of oxo-M, BM 123 and its transformation products for the superhigh affinity site were calculated from their respective IC_{50} values and are given in Table 3.

Figure 10 shows the competitive inhibition of [^{3}H](-)NMS binding by oxo-M, BM 123 and its transformation products. It can be seen that oxo-M and the aziridinium ion have similar IC_{50} values of 6.3 μM and 4.5 μM, respectively, although the shapes of their respective competition curves differed slightly. Both the parent 2-chloroethylamine and the alcoholic hydrolysis product were less active and both had IC_{50} values of approximately 0.32 mM. Nonlinear regression and analysis of variance showed a significant reduction in residual error when the competition curves of oxo-M and BM 123A were fitted to a two-site model as compared to a one-site model. The dissociation constants of the high and low affinity sites (K_H and K_L) and the relative proportion of high affinity sites are given in Table 3. Analysis of variance showed a significant increase in residual error when the oxo-M and BM 123A competition data were fitted by nonlinear regression analysis sharing the estimate of K_L between the data ($F_{2,13} = 9.603$, $P < .01$). The competition curves of the parent 2-chloroethylamine and the alcoholic hydrolysis product were not examined in detail, but their respective IC_{50} values were corrected to give the X_{50} values shown in Table 3.

(8) Kinetics of the alkylation of the superhigh affinity site

The kinetics of muscarinic receptor alkylation by BM 123 were investigated by measuring the binding of both [^{3}H](+)CD and [^{3}H](-)QNB to cortical homogenates that had been incubated with BM 123 for various times. The reaction of BM 123 with the muscarinic receptor was stopped immediately by the addition of atropine (0.1 mM) and thiosulfate (1.0 mM), and the tissue was subsequently washed five times as described in the Materials and Methods. Figure 12 shows the loss of [^{3}H](+)CD binding sites following incubation of cortical homogenates with BM 123 (0.001 - 10.0 μM) for various times. To get a rough estimate of the observed rate constant for alkylation (k_{obs}) at various concentrations of BM 123, the curves shown in Figure 11 were fitted independently to the simple exponential equation:

$$Y = 100 \times e^{-k_{obs}t} \qquad (13)$$

where Y is the percent [^{3}H](+)CD bound and t is time. The estimate of the observed rate constant for alkylation at the various concentrations of BM 123 are plotted in Figure 13 as the open symbols according to the ordinate scale on the left hand side of the figure. The concentration of the aziridinium ion causing a half-maximal increase in k_{obs} was estimated to be 2.8 nM. Receptor occupancy for the aziridinium ion was determined independently by measuring the percent displacement of [^{3}H](+)CD binding to cortical homogenates by BM 123A at 0°. When the percent displacement values are scaled to the maximum value of the observed rate constant and corrected for receptor

TABLE 3

The binding parameters of oxo-M, BM 123 and its transformation products [a].

Compound	K_{SH} (nM)	K_H (nM)	K_L (μM)	X_{50} (μM)	high affinity sites (%)
oxo-M (V)[b]	1.8	19.7	3.06	0.93	31
BM 123A (II)	2.25	12.9	1.23	0.66	25
BM 123 (I)	405			47	
Alcohol (III)	1607			47	

[a] The binding parameters were estimated from the data shown in figures 4 and 5.

[b] The Roman numerals in parentheses refer to the structures of the compounds shown in figure 1.

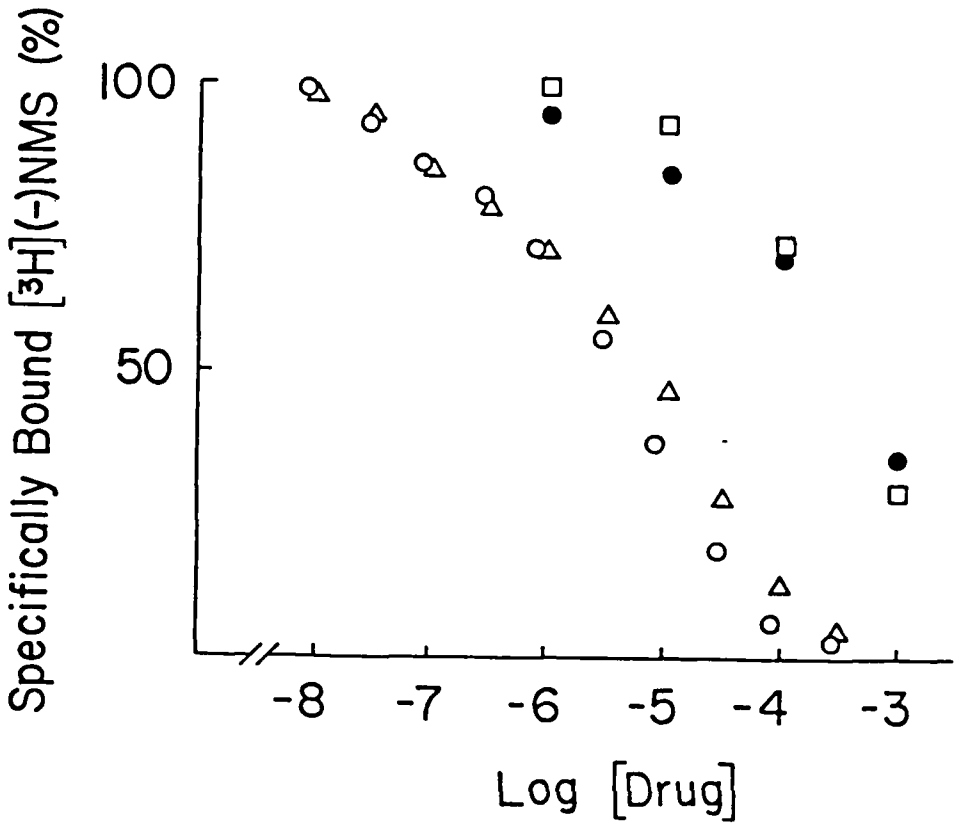

Fig. 10 The competitive inhibition of [^{3}H](-)NMS binding to the rat cerebral cortex by oxo-M (△), BM 123 (□), its aziridinium ion (○) and alcoholic hydrolysis product (●). The specific binding of [^{3}H](-)NMS was measured at 0° in the presence of the indicated concentrations of the various compounds. The concentration of [^{3}H](-)NMS was 0.15 nM. Each data point represents the mean binding value of five experiments, each done in triplicate.

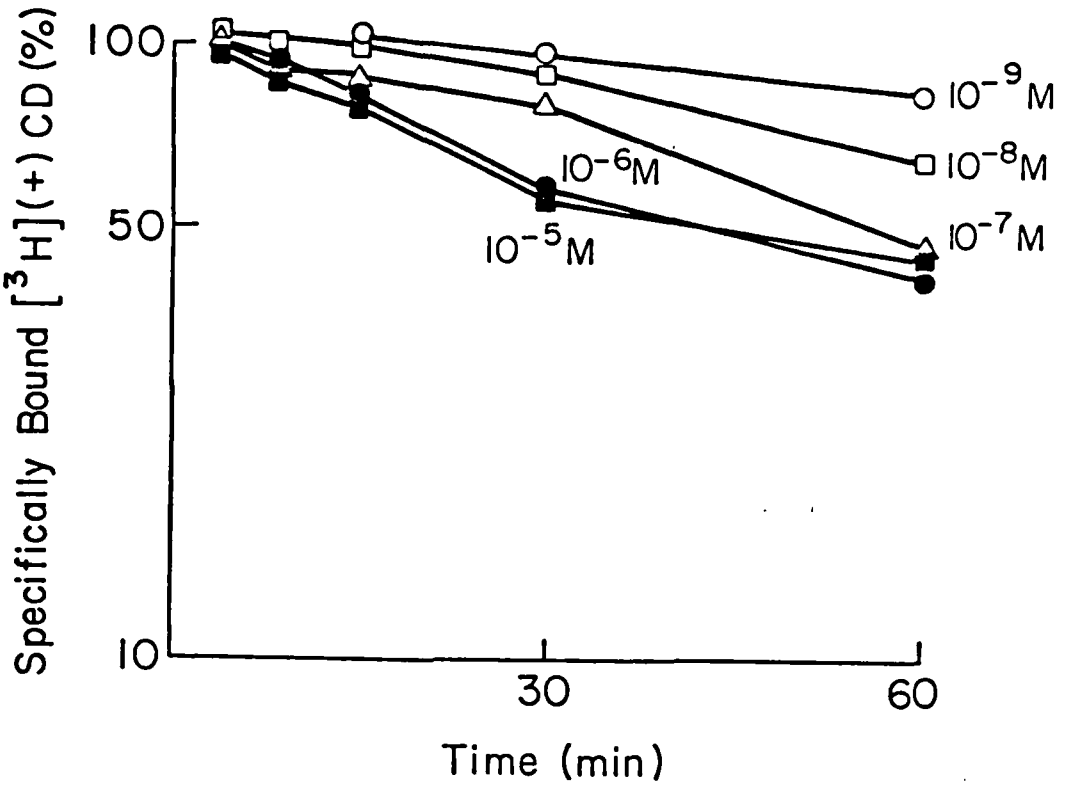

Fig 11 The kinetics of the alkylation of [^{3}H](+)CD binding sites by BM 123 in the rat cerebral cortex.

Cortical homogenates were incubated at 37° with various concentrations of BM 123 for the indicated times, washed extensively, and then assayed for [^{3}H](+)CD binding at 0° using [^{3}H]ligand concentration of 2.0 nM. Specific [^{3}H](+)CD binding was measured in homogenates that had been previously incubated with BM 123 at concentrations at 0.001 μM (○), 0.01 μM (□), 0.1 μM (△), 1.0 μM (●), and 10.0 μM (■). Each point represents the mean binding value of five experiments, each done in triplicate.

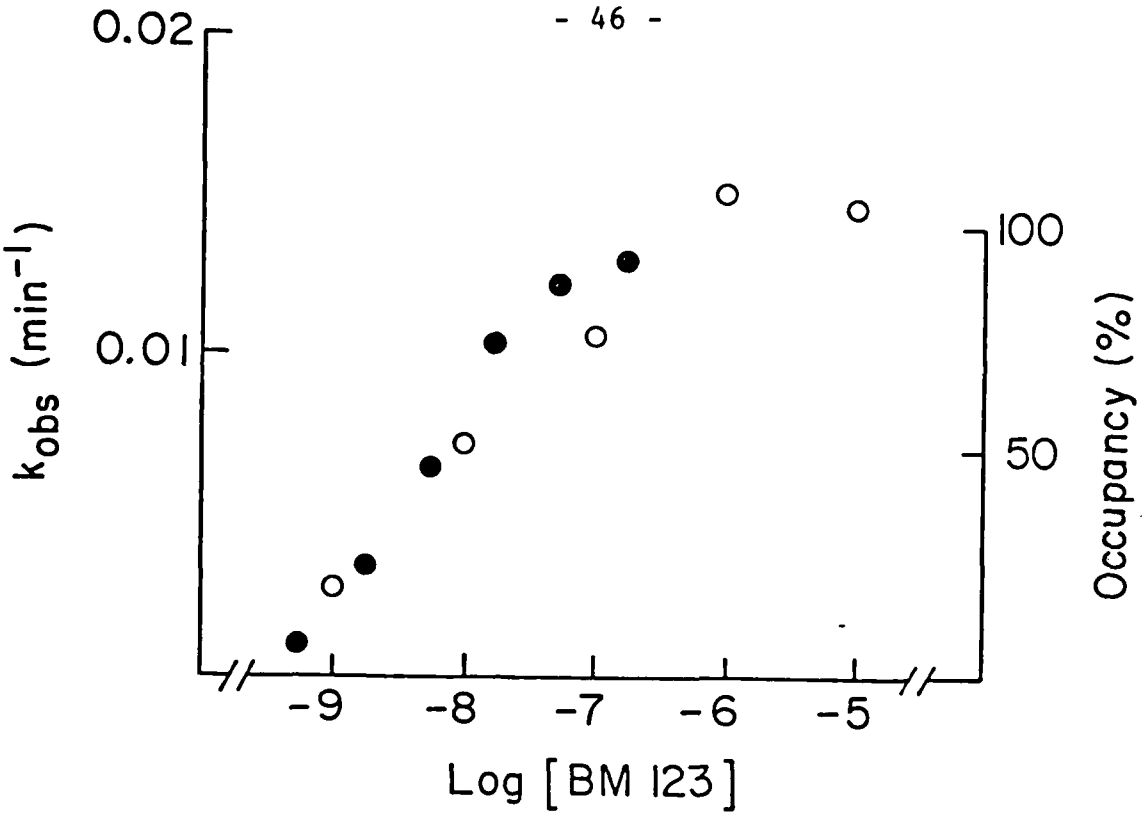

Fig. 12 A relative comparison of the occupancy and the rate of alkylation of [^{3}H](+)CD binding sites by BM 123 at various concentrations.

The observed rate constants for the alkylation of [^{3}H](+)CD binding sites were calculated from the data in figure 6 as described in the text and are plotted in open symbols (○) according to the ordinate scale on the left. Receptor occupancy was determined by measuring percent displacement of [^{3}H](+)CD binding by the aziridinium ion of BM 123 at 0°, and these estimates are plotted with the closed symbols (●) according to the ordinate scale on the right. 100% receptor occupancy has been scaled to the maximum value of the rate constant. The BM 123/ [^{3}H](+)CD competition curve has been corrected for receptor occpancy by [^{3}H](+)CD. The indicated concentrations of BM 123 refer to the initial concentration of the parent 2-chloroalkylamine.

occupancy by [^{3}H](+)CD the resulting occupancy values (see Figure 13; right-hand ordinate scale) agree with the observed rate constants for alkylation. Thus, we conclude that the rate of alkylation is proportional to receptor occupancy. The simplest model to explain the interaction of BM 123A with the [^{3}H](+)CD binding sites is the scheme in equation 1 which is rewritten below in the appropriate terminology for the superhigh affinity site:

$$A + R_{SH} \underset{}{\overset{K_{SH}}{\rightleftharpoons}} AR_{SH} \xrightarrow{k_{SH}} A\text{-}R_{SH} \qquad (14)$$

where R_{SH} is the superhigh affinity site, K_{SH} is the dissociation constant, k_{SH} is the rate constant for alkylation, and AR_{SH} and $A\text{-}R_{SH}$ are the reversible and covalent complexes of the aziridinium ion with the superhigh affinity site, respectively. To test the compatability of the data with the model shown above, the data in figure 6 were fitted simultaneously to equation 5 in the Methods which yielded estimates of K_{SH} and k_{SH} of 2.7 nM and 0.014 min^{-1}, respectively. Analysis of variance showed that the variance estimate based on deviations of the mean binding values from the regression equation was not significantly greater than that estimated by replicate measurements of [^{3}H](+)CD binding, illustrating that the model adequately described the data ($F_{20,92}$ = .178, P > .25).

(9) Kinetics of alkylation of high and low affinity sites

The loss of [^{3}H](-)QNB binding sites following incubation of cortical homogenates with various concentrations of BM 123 is shown in Figure 13. A comparison of the kinetic plots in Figures 11 and 13 shows that the alkylation of [^{3}H](-)QNB binding sites by BM 123 differs from that observed in [^{3}H](+)CD binding experiments in the following ways. Firstly, the rate of loss of [^{3}H](-)QNB sites at high concentrations of BM 123 is much faster than the maximum rate of loss of [^{3}H](+)CD sites. Moreover, the rate of alkylation of the [^{3}H](-)QNB sites continues to increase over a broader concentration range and does not plateau until the BM 123 concentration exceeds 0.1 mM. Finally, the kinetic curves for the loss of [^{3}H](-)QNB sites are more complex, being markedly biphasic at high concentrations of BM 123. This behavior is inconsistent with a simple one-site model, but can be explained by a model in which the aziridinium ion (A) discriminates between high (R_H) and low (R_L) affinity sites, each having its respective dissociation constant (K_H and K_L) and rate constant for alkylation (k_H and k_L):

$$A + R_H \underset{}{\overset{K_H}{\rightleftharpoons}} AR_H \xrightarrow{k_H} A\text{-}R_H \qquad (15)$$

$$A + R_L \underset{}{\overset{K_L}{\rightleftharpoons}} AR_L \xrightarrow{k_L} A\text{-}R_L$$

In this scheme, the aziridinium ion quickly binds with the receptors forming reversible complexes (AR_H and AR_L) which

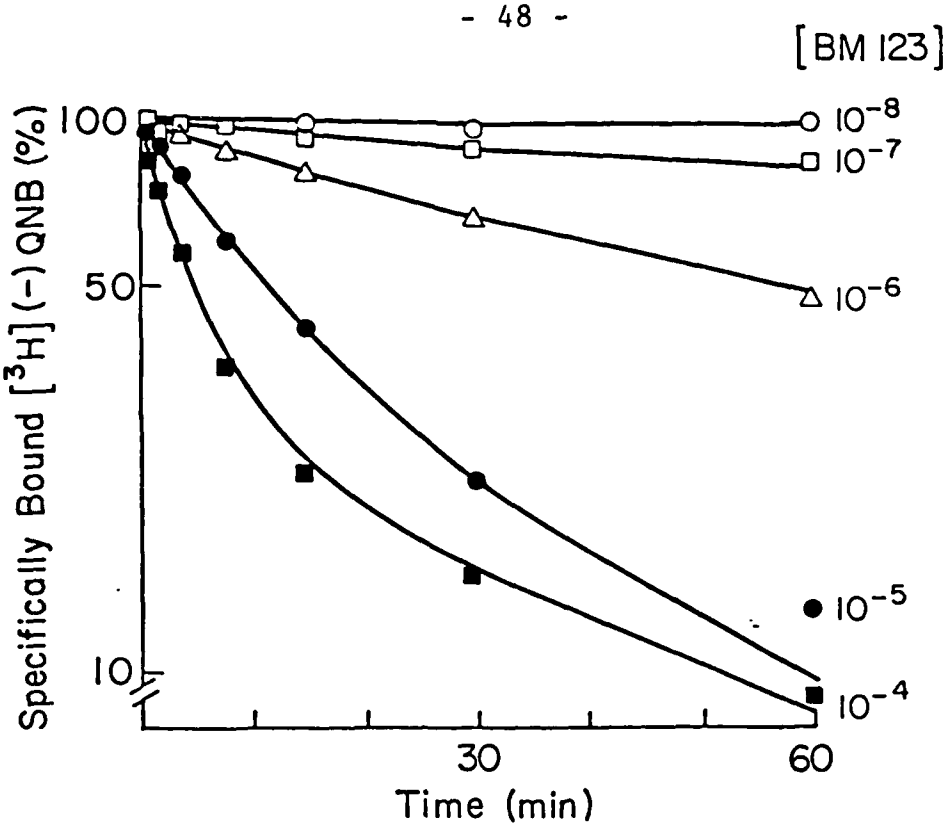

Fig. 13 The kinetics of alkylation of [^{3}H](-)QNB binding site in the rat cerebral cortex by BM 123.

Cortical homogenates were incubated at 37° with various concentrations of BM 123 for the indicated times, washed extensively, and then assayed for [^{3}H](-)QNB binding at 37° using a [^{3}H]ligand concentration of 0.4 nM. Specific [^{3}H](-)QNB binding was measured in homogenates that had been previously incubated with BM 123 at concentrations of 0.01 μM (○), 0.1 μM (□), 1.0 μM (△), 10.0 μM (●) and 100.0 μM (■). Each point represents the mean binding value of six experiments, each done in triplicate.

convert to covalent complexes ($A\text{-}R_H$ and $A\text{-}R_L$) at relatively slower rates. To test the model, the kinetic data were fitted simultaneously to equation 6, the theoretical basis for which is described in Materials and Methods. The results of this analysis showed that BM 123A discriminated between high and low affinity sites having dissociation constants of 18.5 nM and 5.3 M and relative abundances of 30 and 70%, respectively. The rate of alkylation of the high affinity site (k_H = 0.022 min^{-1}) was much slower than that of the low affinity site (k_L = 0.26 min^{-1}). The good agreement between the data and the model can be seen by the theoretical curves in Figure 13 which represent the least squares fit of the data to equation 6. There is some discrepancy between the estimates of the dissociation constants (K_H and K_L) determined kinetically at 37° and those determined at equilibrium by competitive inhibition of [^{3}H](-)NMS binding at 0° (see Table 3). Part of the discrepancy can be attributed to the difference in the incubation temperature of the two experiments, and this issue is discussed below.

To obtain independent estimates of the rate constants for alkylation of the high and low affinity sites, the kinetics of alkylation were examined at a saturating concentration of BM 123 (1.0 mM) so that both sites would be fully occupied by the aziridinium ion, and the observed rate constant for alkylation of each site ($k_{H\ obs}$, $k_{L\ obs}$) should be equivalent to the actual rate constants (k_H, k_L). Figure 14 shows the loss of [^{3}H](-)QNB binding sites following incubation of cortical homogenates with BM 123 (1.0 mM) for various times. The semi-log plot of measurements of [^{3}H](-)QNB binding against time (Figure 15) clearly shows two components. The data in Figure 15 were fitted to the following biexponential equation to estimate the observed rate constants for each component and the relative proportion (a) of the slowly alkylated component:

$$Y = 100\ (ae^{-k_{H(obs)}t} + (1 - a)e^{-k_{L(obs)}t}) \qquad (16)$$

In this equation, Y denotes specifically bound [^{3}H](-)QNB (%). The agreement between the data and equation (16) can be seen by the theoretical curve in Figure 15 which represents the least squares fit to the data. Nonlinear regression analysis showed that 25% of the receptors were alkylated slowly, with an observed rate constant ($k_{H\ obs}$) of 0.016 min^{-1}, whereas the remaining sites were alkylated more rapidly with an observed rate constant ($k_{L\ obs}$) of 0.30 min^{-1}. These estimates of the observed rate constants which were measured at a saturating concentration of BM 123 are in general agreement with the rate constants estimated from the data shown in Figure 14. Moreover, the estimate (25%) of the proportion of sites which are slowly alkylated by BM 123 at a concentration of 1.0 mM is in reasonable agreement with the estimates of the proportion of high affinity sites determined from the kinetic data in figure 8 (30%) and from the equilibrium competition data summarized in Table 3 (25%).

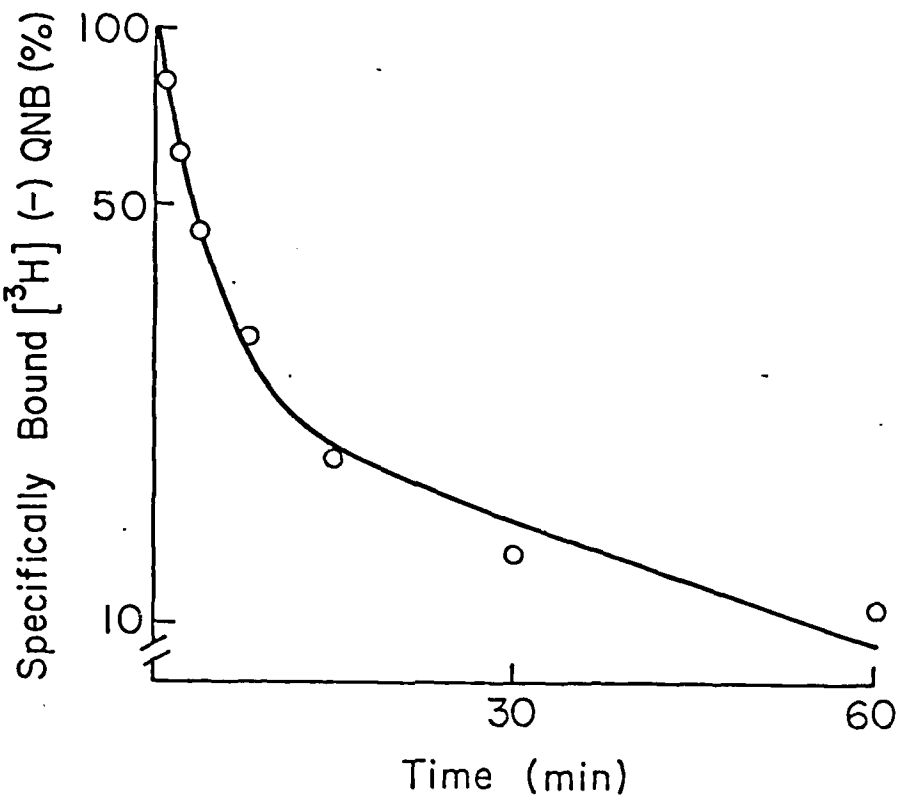

Fig. 14 The kinetics of the alkylation of [^{3}H](-)QNB binding sites in the rat cerebral cortex by BM 123 (1.0 mM).

Cortical homogenates were incubated at 37° with BM 123 for various times, washed extensively, and then assayed for [^{3}H](-)QNB binding at 37° using [^{3}H]ligand concentration of 0.4 nM. Each point represents the mean binding value of four experiments, each done in triplicate.

(10) Selective alkylation of high and low affinity sites

Collectively, the foregoing results are consistent with the postulate that the aziridinium ion discriminates between high and low affinity sites, and that it alkylates the low affinity site at a much faster rate. To test this postulate further, we examined the agonist binding properties of the residual, unalkylated receptors in cortical homogenates which had been treated with BM 123 (1.0 mM) for 10.3 min. This time of incubation with BM 123 corresponds to the bend in the semi-log plot shown in Figure 14. Using the parameter values estimated above, it can be calculated that, at this point during the incubation, 95% of the low affinity sites had been alkylated with BM 123 whereas only 15% of the high affinity sites had been alkylated. Figure 15A shows the results of oxo-M/[^{3}H](-)NMS competition curves run on control tissue and tissue which had been previously incubated with BM 123 (1.0 mM) for 10.3 min and washed extensively. Nonlinear regression analysis showed that the control competition curve was compatible with the existence of high (K_H = 0.028 μM) and low (K_L = 10.6 μM) affinity sites having relative abundances of 30 and 70%, respectively. Following BM 123 treatment, 65% of the receptors were alkylated, and there was a 25-fold reduction in the X_{50} value of oxo-M from 3.0 μM in control homogenates to 0.12 μM in BM 123 treated homogenates. Nonlinear regression analysis showed that this shift in the oxo-M/[^{3}H](-)NMS competition curve could be attributed to a selective increase in the proportion of high affinity sites to a value of 70%. Similar results were observed when the competitive inhibition of [^{3}H](-)NMS binding by carbachol was studied in control and BM 123 treated (1.0 mM; 10.3 min) tissue (Figure 15B). Nonlinear regression showed that the control carbachol/[^{3}H](-)NMS competition curve was consistent with the presence of high (K_H = 0.21 μM) and low (K_L = 49 μM) affinity sites having relative abundances of 27% and 73%, respectively. Following BM 123 treatment, there was a 25-fold reduction in the X_{50} value of carbachol from 25 μM in controls to 1.0 μM in BM 123 treated homogenates, and an increase in the proportion of high affinity sites to a value of 66%.

In contrast, prior treatment of cortical homogenate with BM 123 (1.0 mM) for 10.3 min had no significant influence on the ability of atropine (X_{50} = 0.80 nM) and gallamine to inhibit [^{3}H](-)NMS binding to the residual receptors (Figure 16). A high concentration (1.0 mM) of gallamine did not fully displace [^{3}H](-)NMS binding; consequently, the gallamine inhibition curve plateaued at 80% inhibition of [^{3}H](-)NMS binding. Stockton et al. (58) have shown that gallamine does not directly compete with [^{3}H](-)NMS for the same site, but rather, it binds at an allosteric locus to reduce the affinity of [^{3}H](-)NMS for the primary site. By fitting the gallamine/[^{3}H](-)NMS inhibition curve to the appropriate equation given by Stockton et al. (58), we estimated that the dissociation constant of gallamine for its site to be 5.1 μM in the absence of [^{3}H](-)NMS. A small but significant increase in the concentration of pirenzepine required to inhibit [^{3}H](-)NMS binding by 50% was observed following

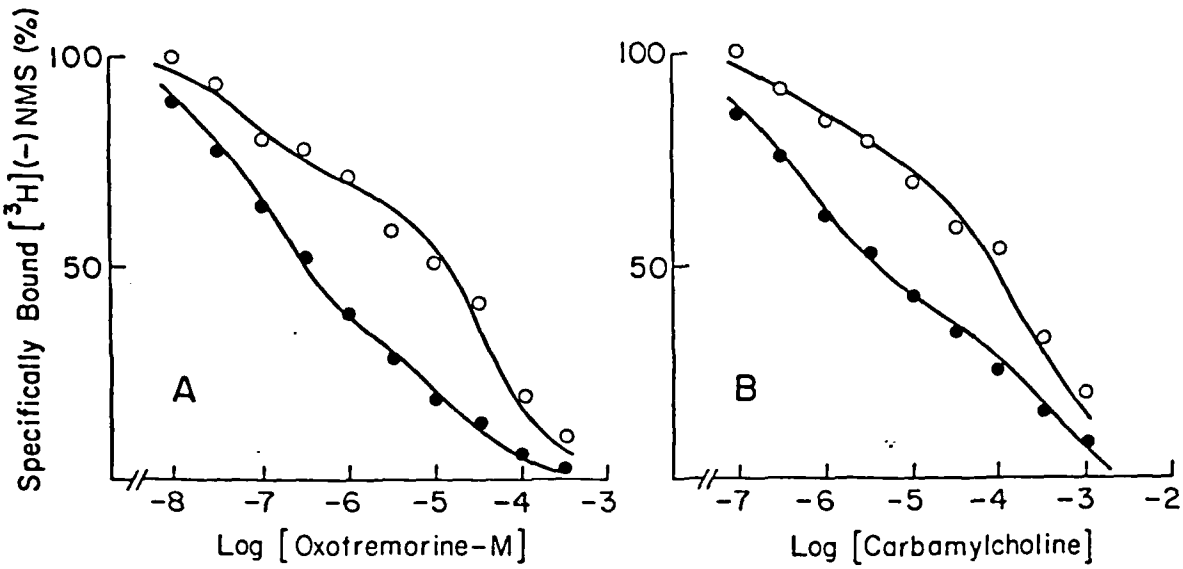

Fig. 15. The competitive inhibition of $[^3H](-)$NMS binding by oxo-M (A) and carbamylcholine (B) in control homogenates (open symbols) and homogenates which had been incubated at 37° with BM 123 (1.0 mM) for 10.3 min and washed extensively (closed symbols).

The data points represent the mean binding value of 3 - 4 experiments, each done in triplicate. The incubation of tissue with $[^3H](-)$NMS (0.15 nM) lasted 30 min at 30°.

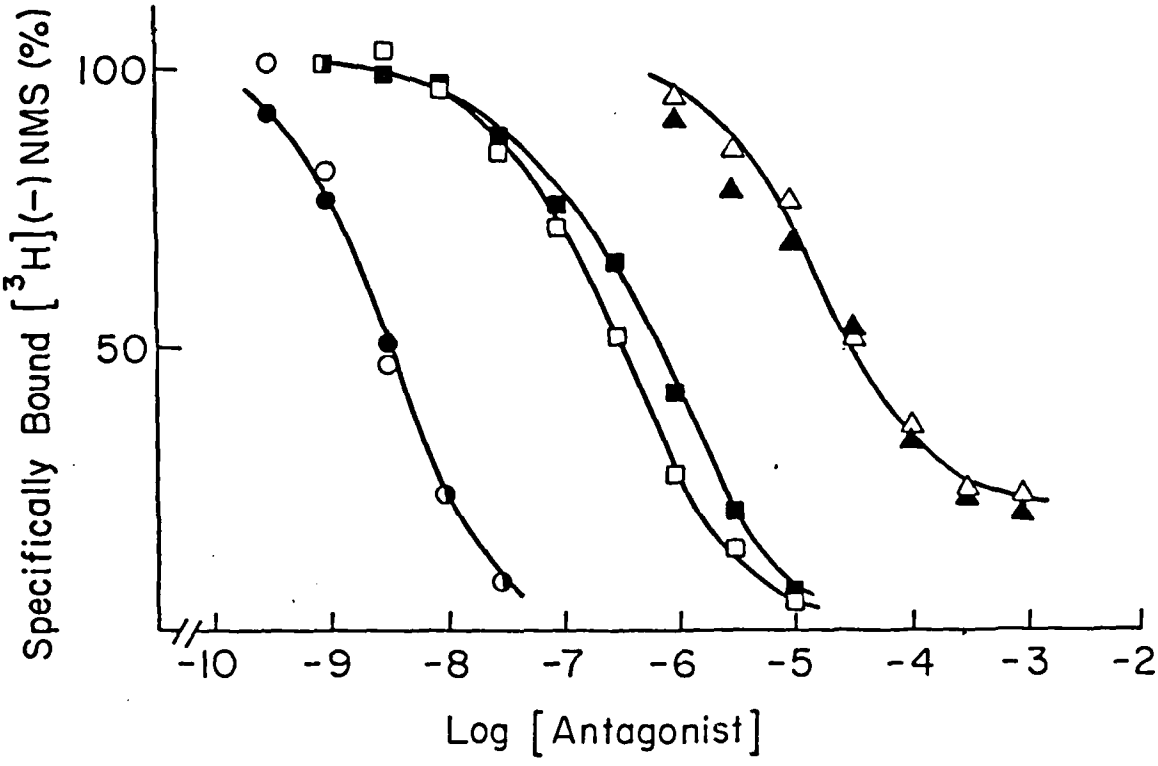

Fig. 16. The inhibition of [^{3}H](-)NMS binding by atropine (circles), pirenzepine (squares), and gallamine (triangles) in control homogenates (open symbols) and homogenates which had been at 37° incubated with BM 123 (1.0 mM) for 10.3 min and washed extensively (closed symbols).

The data points represent the mean binding values of three experiments, each done in triplicate. The incubation of tissue with [^{3}H](-)NMS (0.15 nM) was 30 min at 30° for the experiments with atropine and pirenzepine and 3 hr for the experiments with gallamine.

incubation of cortical homogenates with BM 123 (1.0 mM) for 10.3 min (Figure 16). The X_{50} value of pirenzepine increased from 80 nM in controls to 142 nM in BM 123 treated homogenate.

Since the ratio of dissociation constants (K_L/K_H) of the aziridinium ion for the high and low affinity sites was greater than the ratio of alkylation rate constants (k_L/k_H) for the two sites, it seemed likely that we could selectively alkylate the high affinity site by incubating cortical homogenates with a low concentration of BM 123 (0.1 μM) for a relatively long time (1 hr). Figure 17 shows the results of oxo-M/[^{3}H](-)NMS competition experiments run of control homogenates and homogenates which had been incubated with BM 123 (0.1 μM) for 1 hr. Nonlinear regression analysis showed that the control competition curve was compatible with the presence of high (K_H = 28 nM) and low (K_L = 4.4 μM) affinity sites having relative abundances of 34 and 66%, respectively. Following treatment with BM 123 (0.1 μM) for 1 hr, 28% of the receptors were alkylated, and there was a small but significant ($P < 0.01$) 1.9-fold increase in the X_{50} of oxo-M from 1.3 μM in controls to 2.5 μM in BM 123 treated homogenates. Regression analysis showed that it was possible to explain this shift as being the result of a small decrease in the proportion of high affinity sites, the proportion of high affinity sites being 23% in the BM 123 treated tissue.

(11) Competitive inhibition of alkylation

Experiments were run to determine if there was agreement between the potency of various reversible muscarinic agents for competitively slowing the rate of receptor alkylation by BM 123 and their respective potencies for interacting with muscarinic receptors. Figure 18A shows the results of experiments in which [^{3}H](-)QNB binding was measured in extensively washed homogenates that had been previously exposed to BM 123 (10 μM) in combination with various concentrations of atropine. It can be seen that nanomolar concentrations of atropine slowed the rate of receptor alkylation by BM 123 and that the rate of receptor alkylation decreased with increasing atropine concentration. The simplest scheme to illustrate the competitive inhibition of receptor alkylation is:

$$\begin{array}{ccccc} A + I + R & \overset{K_A}{\rightleftharpoons} & AR & \longrightarrow & A\text{-}R \\ & \underset{K_I}{\searrow\!\!\nearrow} & & & \\ & A + IR & & & \end{array} \qquad (17)$$

In this scheme, K_A and K_I are the dissociation constants of the aziridinium ion (A) and competitive inhibitor (I), respectively, AR and IR are reversible receptor complexes, and A-R is the covalent receptor complex. If we make the simplifying assumptions that the concentrations of I and A are constant during the reaction and that the rate of the alkylation is slower than that of the reversible interactions, then it can be shown that:

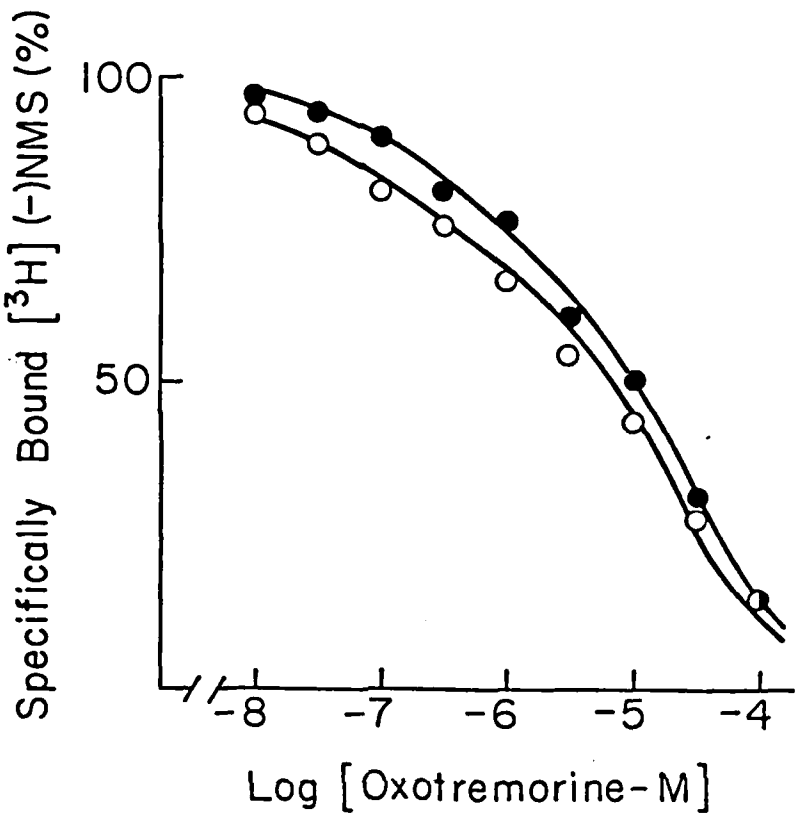

Fig. 17. The competitive inhibition of $[^3H](-)$NMS binding by oxo-M in control homogenates (O) and homogenates which had been incubated at 37° with BM 123 (0.1 μM) for 1 hr and washed extensively (●).

The data points represent the mean binding value of four experiments, each done in triplicate. The incubation of tissue with $[^3H](-)$NMS (0.15 nM) lasted 30 min at 30°.

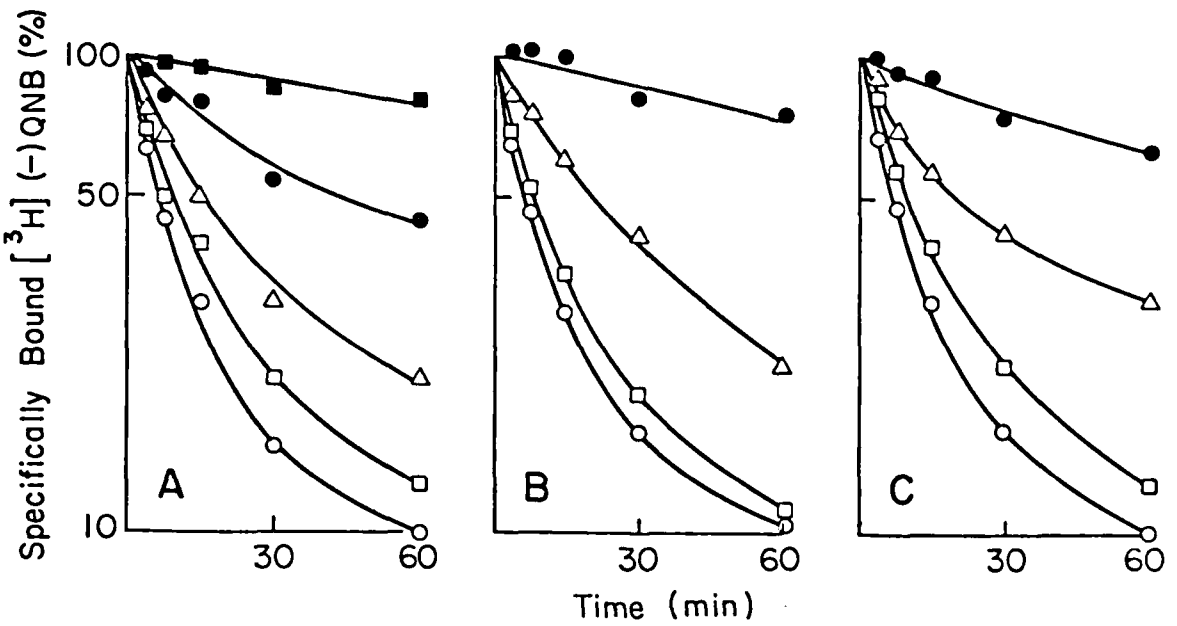

Fig. 18. The effects of atropine (A), oxotremorine (B) and oxo-M (C) on the rate of alkylation of [^{3}H](-)QNB binding sites in rat cerebral cortex by BM 123 (10 μM).

Cortical homogenates were incubated at 37° with BM 123 for the indicated times in the absence (C) and presence of various concentrations of atropine: 1.0 nM (□), 3.0 nM (△), 10 nM (●) and 100 nM (■); oxotremorine: 0.3 μM (□), 3.0 μM (△) and 30.0 μM (●); and oxo-M: 1.0 μM (□), 10 μM (△) and 100 μM (●). The homogenates were then washed extensively and assayed for [^{3}H](-)QNB binding at 37° using a [^{3}H]ligand concentration of 0.4 nM. The data points represent the mean binding value of two experiments, each done in triplicate.

$$R - 1 = I/K_I' \quad (18)$$

where R is the ratio of the half-times for alkylation of muscarinic receptors in the presence of I divided by that measured in the absence of I, and K'_I is the apparent dissociation constant of the competitive inhibitor ($K'_I = K_I(1 + A/K_A)$). In logarithmic form, equation (18) is a straight line having a slope of one and an x-intercept equal to log K'_I:

$$\log (R - 1) = \log (I) - \log (K_I') \quad (19)$$

The competition data for atropine (Figure 18A) and similar data for oxotremorine and oxo-M (Figure 18B,C) were plotted according to equation (19) above, and the results of this analysis are shown in Figure 19. The estimates of the apparent dissociation constants for atropine, oxotremorine and oxo-M were 2.5 nM, 1.0 μM and 4.0 μM, respectively.

4.1.9 Recovery of muscarinic receptors

A clear understanding of homeostatic receptor regulation in vivo may provide insight into mechanisms of drug tolerance or supersensitivity and perhaps suggest ways of manipulating receptor numbers for therpeutic benefit. The time course of receptor recovery from irreversible blockade must ultimately depend on these basic parameters of receptor regulation.

N-[4-(2-chloroethylmethylamino)-2-butynl]-2-pyrrolidone (BM 123) was injected into rat tail veins in a dose schedule which blocked more than 90% of cortical and striatial muscarinic receptors (8, 20, and 50 μmoles kg^{-1} at 1 hr intervals). Animals were decapitated at later time points and densities of unalkylated receptors present were determine by specific (10^{-6} M atropine displaceable) binding of 0.4 nM ^{3}H-l-quinuclidinyl benzilate (QNB) to well-washed membrane fragments from homogenates of cerebral cortex, striatum, and longitudinal muscle of the ileum. The binding incubation was conducted in 50 mM Na/K PO_4, pH 7.4, for 1 hr at 37°C. Rapid filteration through GF/B filters and 3 washes with cold saline terminated the assay. Receptor densities were quantified in terms of fmoles/mg protein.

The recovery time course (Figure 7) in each tissue found to approximate a singel exponential curve described by this model:

$$R_t = R_{ss} - R_a e^{-kt}$$

R_t is free receptor density at time, t(hrs), as measured by ^{3}H-QNB binding. R_{ss} is steady state receptor density which was found to be the same before (control tissue) and after alkylation. R_a is the alkylated receptor density, extrapolated to t=0, and k is the degradation rate constant (hr^{-1}). This model assumes (a) a constant rate of receptor synthesis; (b) first order rate of receptor degradation; (c) the R_{ss} after recovery is the same as R_{ss} before alkylation.

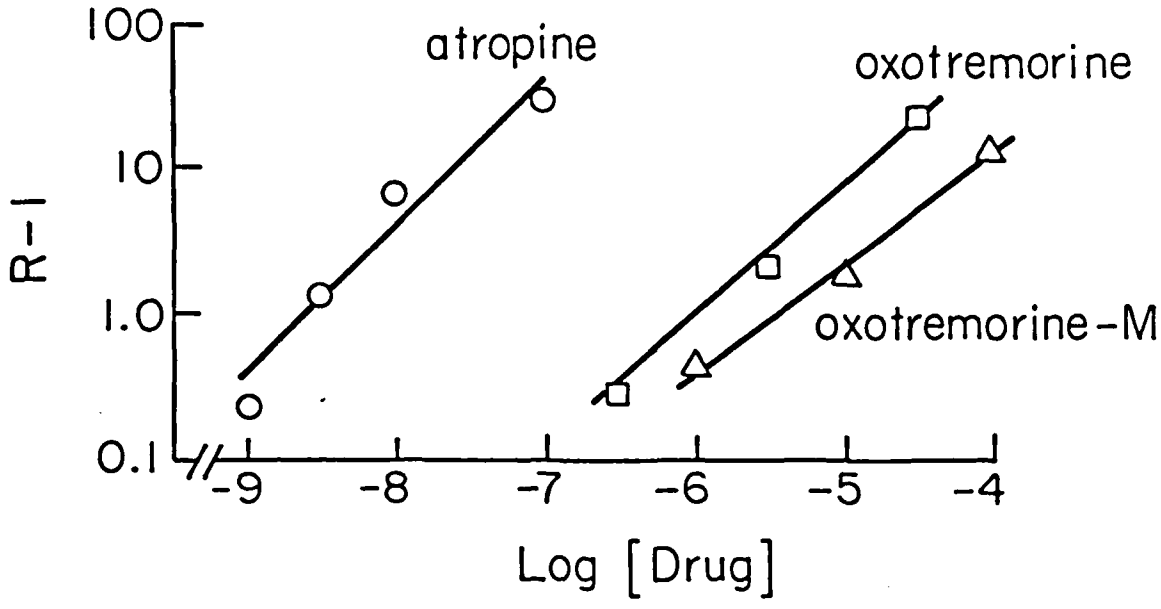

Fig. 19. The effects of atropine, oxotremorine and oxo-M on the rate of alkylation of [^{3}H](-)QNB binding sites by BM 123 (10 μM).

R refers to the ratio of half-times for the alkylation of [^{3}H](-)QNB binding sites by BM 123 in the presence of the inhibitor divided by that measured in the absence of the inhibitor. The R values were calculated from the data shown in figure 13. The straight lines represent the least squares fit to the data for atropine (slope = 1.01), oxotremorine (slope = 0.94) and oxo-M (slope = 0.72).

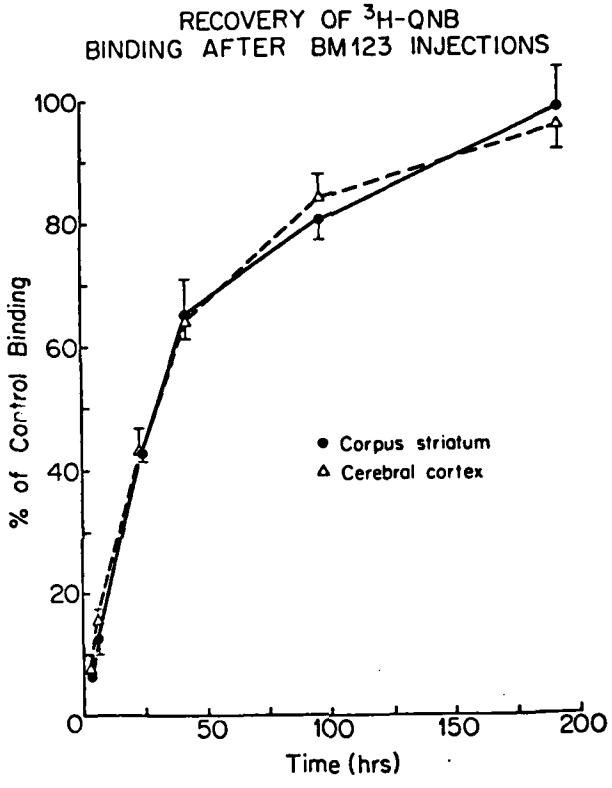

Fig. 20. Recovery of [^{3}H](-)QNB binding after BM 123 injections

Each true point upon n = 3 rats; n = 15 for control subjects. Standard errors were calculated by the variance of ratio method. At some points standard errors were too small to be plotted.

The model was fitted to recovery curves by least squares non-linear regression yielding a best fir rate constant, k, of $0.021 \pm .001\ hr^{-1}$ (half-time 33 hrs) in all 3 tissues. Since the degradation rate constant was found by ANOVA to be identical in all 3 tissues, the different steady state densities in these tissues are likely to be different, tissue-dependent, rates of receptor synthesis.

4.1.10 Acetlycholine Release

There is strong pharmacological evidence for the presence of inhibitory muscarinic receptors located presynaptically both in the periphery and in the central nervous system (CNS) (59,60). The evidence has come from experimentation using such preparations of CNS tissue as cortical slices, synaptosomal fractions, and cell-free suspensions of nerve endings and from experiments on the myenteric plexus. These studies have involved spontaneous ACh release and release evoked by electrical or K^+ stimulation. A generalization from the results is that cholinergic agonists have an inhibitory action and cholinergic antagonists a stimulating action on ACh release in these preparations. Such findings have led to the hypothesis that feedback regulation of ACh release is exerted by way of muscarinic presynaptic receptors which are located on the cholinergic nerve ending and therefore act as autoreceptors (61).

Such a feedback mechanism could be involved in the development of tolerance to anticholinesterase agents, e.g. DFP. Following treatment with DFP an increase in the half-life of ACh could serve as the pacemaker step in the series of events associated with both acute and chronic effects of the agent, including effects on behaviors coded cholinergically (18). Inhibition of ChE could increase the proportion of ACh escaping from the synaptic region by diffusion (62) and prolong the half-life of the fraction which escaped. Thus, significant levels of ACh could be maintained at some sites at steady state following DFP treatment. It is not implausible that chronic treatments could result in adaptive changes in receptor concentrations at presynaptic as well as postsynaptic muscarinic sites. If presynaptic autoreceptors down-regulate, this would be expected to lead to disinhibition of ACh release, a non-adaptive response. To be adaptive ACh release should be progressively inhibited as ACh levels rise.

The present lack of a specific, high affinity ligand for presynaptic mAChRs prevents the testing of these possibilities by the radioligand procedures. However, it is possible to study changes in ACh release following acute and chronic DFP treatment using the myenteric plexus preparation. It is known that strips of the ileum release ACh if incubated in eserinized solutions (63). Further evidence (64) has demonstrated that the ACh metabolism of such strips is a property of Auerbach's plexus, which innervates the longitudinal muscle. More recently the longitudinal muscle/myenteric plexus preparation has been adopted as a peripheral model for studying muscarinic modulation of ACh

release (65,66,67). We have used it in the experiments described below.

(1) Animals

Sprague-Dawley male rats (Simonsen, Gilroy CA) served as subjects. They weighed between 180 and 225 g on entry into the laboratory. During the course of the experiments they were housed in individual cages in a temperature and humidity controlled room with a 12/12 hr light-dark cycle. Each was randomly assigned to its respective treatment group. Food and water were available ad libitum. All animals were habituated to the laboratory and their living conditions for at least one week before participating in experiments. On the day preceding their first injections they were handled for 2 min and weighed.

(2) DFP treatment

On DFP treatment day 1 all animals were taken from their cages, weighed and returned to the cages. DFP subjects were then injected with DFP in arachis oil and control animals with arachis oil only. Injections were scheduled so that sacrifice for assays of the myenteric plexus could take place at 8:00 a.m., a limitation imposed by the time required for the assay and by the diurnal changes in levels of ACh (68). For all except the 4 and 12 hr acute groups injections were administered at approximately 8:00 a.m., the start of the daily light cycle.

All injections were subcutaneous in a standard volume of 1.0 ml kg^{-1} body weight. The regimen for DFP subjects was selected after preliminary tests and was similar to that used by (23) in their studies of effects of DFP on contractile responsiveness and mAChR parameters in the longitudinal muscle of the ileum. On the first treatment day experimental animals received injections of 2.0 mg kg^{-1} DFP. After a 72 hr period of no treatments chronic subjects were administered 0.5 mg kg^{-1} DFP daily until their full complement, i.e. 2, 4 or 8, was reached in a total of 4, 6, or 10 days. These durations of chronic treatment were chosen on the basis of earlier reports that by 10 days tolerance has developed, as indicated by the disappearance of parasympathomimetic signs and return to pretreatment baselines of a wide variety of behavior patterns (1,17,69). All chronic subjects were sacrificed 24 hr after their last injection. Paired control groups were given arachis oil on identical schedules. The chronic 10-day treatment and its arachis oil control were replicated in an independent experiment as a check on the original findings.

(3) Myenteric plexus

After completion of their respective treatments with DFP or arachis oil, the animals were sacrificed by decapitation and ileum and brain removed. The latter was frozen immediately and kept at -20°C until assayed for AChE activity. The longitudinal muscle with associated myenteric plexus was dissected

approximately as described by (70). Four ileum pieces approximately 4.0-4.5 cm long were removed from the animal and the longitudinal muscle was carefully teased from its underlying circular layer. Each strip weighed approximately 30 mg. The strips were then immersed in Tyrode solution (composition: NaCl 137 mM; KCl 2.7 mM; $CaCl_2$ 1.8 mM; $MgCl_2$ 1.0 mM; NaH_2PO_4 0.4 mM; $NaHCO_3$ 11.9 mM; glucose 5.6 mM), equilibrated with 95% O_2, 5% CO_2. The medium also contained 30 μM eserine to prevent ACh hydrolysis and 10 μM EDTA. The strips were mounted on electrodes consisting of 2 platinum wires 12 mm apart. The muscle was tied at the base with silk thread, then looped around the second wire and attached to the base again. Thus, the strip was stimulated directly between the two electrodes. The electrodes with attached longitudinal muscle were then immersed in 3 ml Tyrode solution containing 30 μM eserine and allowed to equilibrate for 1 hr, the medium being changed every 15 min.

The experimental design paralleled that of Kilbinger (65,66). Each 30 min treatment period followed the same pattern: a 10 min resting phase; 10 min stimulation phase; 10 min recovery phase. The strip was exposed to the various atropine concentrations during the 10 min resting and stimulation, with no drug during the recovery phase. Preliminary experiments showed that longer incubations produced essentially identical results. At the end of the final treatment period of some experiments, the tissue was removed from the electrodes, rinsed in cold saline and analyzed for ACh and Ch levels by GCMS. In other experiments the AChE activity was measured in the tissues.

The treatment periods began with 2 control measurements, which provided information about pre-atropine (basal) rates of release. These were followed by 5 exposures to atropine at concentrations of 10^{-9} to 10^{-5} M, in order from low to high concentrations. At the end of each 10 min time phase the electrodes and tissue were transferred to the next tube and the entire 3 ml contents were analyzed for ACh and Ch as described by Jenden et al. (71) and Freeman et al. (72).

Stimulation parameters were: frequency, 3 Hz; duration, 1 msec; current, 200 mA. This current strength is supramaximal with respect to ACh release.

(4) Acetylcholinesterase (AChE)

AChE activity was measured by the colorimetric method described by Ellman et al. (73).

(5) Statistical Analyses

All ANOVAs took into consideration that repeated measures on the same subjects were involved. They were carried out using the BMDP2V program, "Analysis of Variance and Covariance with Repeated Measures", provided by BMDP Statistical Software, Inc. (Westwood, CA), the computations being performed in double precision. When ANOVA provided evidence of significant effects

at levels of confidence of 10^{-2} or better, post hoc tests to identify the specific bases for the effects were carried out using the Scheffe (74) procedure for multiple comparisons. The level of confidence, 10^{-2}, was chosen in order to minimize the risk of misinterpretations were a less stringent criterion used when many tests for significance were performed.

(7) Materials

Diisopropylfluorophosphate was obtained from Sigma Chemical Co., St. Louis, Missouri; atropine sulfate was from Mallinckrodt Chemical Co., Paris, Kentucky; and eserine sulfate (physostigmine) from Calbiochem, San Diego, California.

(8) Signs

All animals treated with DFP showed the acute signs of intoxication to be expected when brain AChE activity is suppressed and ACh levels elevated. They developed tremor and parasympathomimetic signs including diarrhea, urination, piloerection, chromodacryorrhea (poyphyrin secretion from the lacrmial gland) and excessive salivation, indicating that both central and peripheral mechanisms were involved. The signs decreased in magnitude and frequency during chronic treatment and were not observable after the third injection. None of the control group showed any of the signs.

(9) AChE activity levels

Determination of AChE activity levels was essential as a control for the potency of the DFP administered. Previous experience had shown that marked variations in potency may occur from sample to sample of that agent. What may appear to be pharmacodynamic effects may merely reflect pharmacokinetic differences associated with variations in potency. In addition to checking behavioral effects of test doses prior to the main experiments, AChE activity levels were determined for a random sample of whole brains after sacrifice of animals subjected to the various experimental treatments. Mean levels following acute treatment by 4, 12 and 24 hrs, expressed as percent of arachis oil control subjects were 15.9%, 18.9%, and 19.8%, respectively. Mean levels among animals treated with DFP chronically for 4, 6 and 10 days were 18.7%, 20.3% and 15.5%. The mean AChE activity level for all groups combined was 18.0% with a standard error of 1.42%. ANOVA including all DFP-treated groups produced an $F_{(5,15)}=0.31$, $p > 5 \times 10^{-2}$. These levels were similar in magnitude to those reported by other investigators using a comparable regimen (75). Earlier experiments had shown that similar DFP regimens produce rapid decreases of AChE activity to levels which are maintained during the course of treatment (76,77). AChE activity was also measured in randomly selected myenteric plexus preparations from controls and from animals treated with DFP for 10 days. The mean level in the DFP-treated animals was 22.3±2.1% (n = 10).

(10) Resting ACh Release

ANOVA of ACh release during the resting phases which preceded the 10 min periods of stimulation showed no significant differences among the seven DFP treatment groups: $F_{(6,59)}=2.30$, $p > 5 \times 10^{-2}$. However, within-group ANOVA for repeated measures was significant, $F_{(6,354)}=14.23$, $p < 10^{-10}$, indicating a systematic trend toward decreasing release as the exposures to atropine progressed. The trend was evidenced in Scheffe analyses by increasingly significant differences between initial and subsequent rates of release. The mean resting release rate for all strips prior to atropine treatment was 158±19 pmol g^{-1} min^{-1}. The mean resting release rate in the final period (10^{-5} M atropine) was 114 pmol g^{-1} min^{-1}, or 28% less than initially. This effect was seen primarily after the shortest exposures to DFP (4 and 12 hr). In contrast, experiments of the same design in which no atropine was added showed no significant differences among exposure times, either for an arachis oil control ($F_{6,42}=1.56$, $p = 1.8 \times 10^{-1}$) or for an experiment carried out after 10 days of exposure to DFP ($F_{6,18}=1.71$, $p = 1.8 \times 10^{-1}$). During the final measurement period the release was -7% and +1% when compared with the first two periods. It must be concluded that atropine causes a small decrease in resting release; however, this effect is very small compared to the substantial increases in evoked release caused by atropine (see below).

(11) Evoked ACh Release: Basal Rates

Evoked ACh release (differences between stimulated and resting release for each of the strips of myenteric plexus studied) was used as the primary measure for determining effects of the several treatments. Basal rate was defined as evoked release in the absence of atropine. ANOVA for differences between the various DFP treatment groups showed that there was a highly significant overall effect: $F_{(6,53)}=4.94$, $p = 4.4 \times 10^{-4}$. Further analyses using the Scheffe test for multiple contrasts indicated that there was no significant difference between the arachis oil control strips and those from animals assayed 4, 12 and 24 hr after a single injection. It was clear from the Scheffe comparisons that the significant overall F test was due to a higher rate of evoked release of strips from the chronically-treated compared to strips from control animals. Multiple contrasts also showed that there were no significant differences among the 4, 6 and 10 day groups. These results were confirmed by the replication of arachis oil and 10-day chronic treatments, comparison of these groups using a paired t test yielding a $t_{14}=7.35$, $p < 10^{-5}$. Similar analysis of data from the original experiment resulted in $t_{33}=4.64$, $p < 10^{-4}$. The evoked ACh release rates are summarized in Table 4.

The stability of measures of basal evoked release was tested by separate experiments using strips from control animals and those receiving the 10 day DFP treatment. Release was measured during repetition of the standard experimental conditions, but without addition of atropine. In preparations from DFP-treated

animals there were no significant differences in level of evoked release during the seven successive measurements: $F_{(6,18)}=2.80$, $p > 10^{-2}$. In preparations from control animals a significant difference was observed ($F_{6,42}=8.81$, $p = 3.1 \times 10^{-6}$) which by the Scheffe criterion was attributable to a small increase (19%) from the first two stimulation periods (mean 442 pmol g^{-1} min^{-1}) to the last five (mean 525 pmol g^{-1} min^{-1}). There was no consistent trend and regression on time was not significant.

(12) Tissue Levels

Levels of Ch and ACh were measured in myenteric plexus at the end of most experiments. Analysis of variance revealed no significant differences between DFP treatment groups for Ch ($F_{5,54}=2.10$, $p = 7.9 \times 10^{-2}$) or ACh ($F_{5,54}=1.31$; $p = 2.7 \times 10^{-1}$). The mean levels were 36.3±2.0 (n = 60) and 47.9±2.0 (n = 60) nmol g^{-1}, respectively.

(13) Evoked ACh Release: Atropine Dependency

Figure 21 presents a graphic summary of effects of the arachis oil control and the chronic DFP treatments on evoked release at different concentrations of atropine. The graph shows substantial atropine dose dependencies in ACh release for both treatment groups. The general shape of the curve for the control condition is identical with similar dose-effect curves found for controls in other experiments in our laboratory. ANOVA (within groups) for repeated measures established that the atropine had a highly significant effect on release rate: $F_{(6,318)}=121.01$, $p < 10^{-10}$. Results of the replication involving only the 10-day chronic control and DFP treatments confirmed the significance of this trend: $F_{(6,84)}=60.18$, $p < 10^{-10}$.

The curves in Fig. 21 suggest that, although they are displaced at all atropine concentrations, increases in rates of evoked ACh release with increasing concentrations of atropine were similar. Scheffe contrasts for differences between release rates at atropine concentrations of 10^{-9} and 10^{-7} M, $(10^{-7})-(10^{-9})$, showed that this was, in fact, the case, i.e. the slopes of the two curves were the same within this range.

ANOVA showed that there also were significant differences between the control and the various DFP-treated groups: $F_{(6,53)}=2.97$, $p < 10^{-2}$. Further analyses using the Scheffe procedure established that these effects were due to greater ACh release by the chronically-treated groups, there being no significant differences between the acute and arachis oil control treatments. A 2-way ANOVA of the chronic groups only provided evidence that these treatments did not differ significantly from each other, $F_{(2,18)}=0.10$, $p > 10^{-2}$, and that their combined effects were highly atropine dose dependent, $F_{(4,72)}=35.15$, $p < 10^{-10}$. In none of the analyses was there a significant interaction between the DFP and atropine variables. Rates of evoked release are shown in Table 4.

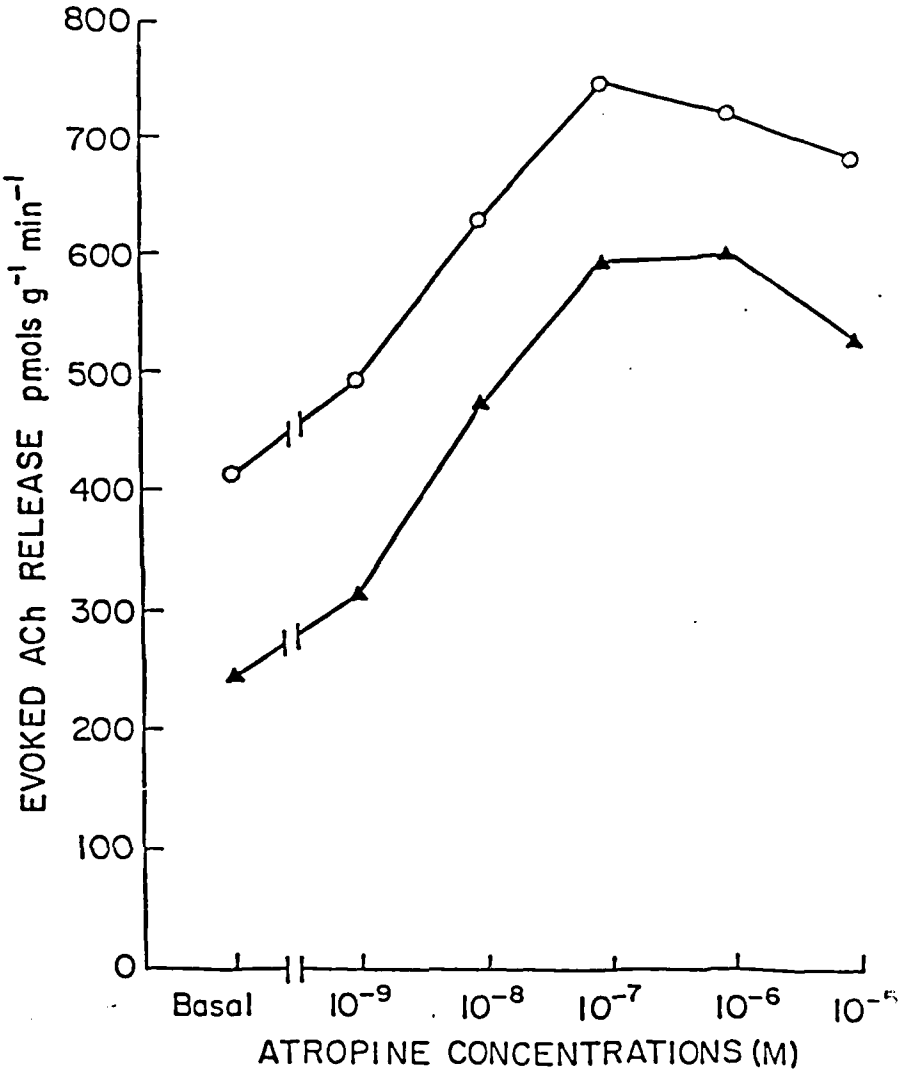

Fig. 21 Evoked ACh release: basal levels and atropine dependencies

Evoked release in the absence of atropine (basal level) was significantly greater ($p < 10^{-3}$) in strips of myenteric plexus from animals treater chronically with DFP (open circles) than in strips from saline control animals. Increase in rates of evoked release with increasing concentrations of atropine were similar and the trends highly significant ($p < 10^{-10}$).

TABLE 4

ACh Evoked Release Rates in pmol $g^{-1}min^{-1}$: Mean±SEM

Treatment	n	Basal (preatropine)	Atropine Concentration (M) 10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Arachis oil	24	243 ±19	316 ±23	473 ±33	593 ±44	601 ±50	529 ±45
DFP 4hr	4	232 ±54	282 ±88	389 ±128	487 ±122	478 ±126	474 ±119
12hr	4	149 ±41	190 ±42	327 ±84	376 ±128	331 ±101	334 ±103
24hr	7	336 ±59	404 ±66	556 ±107	596 ±96	584 ±88	546 ±98
DFP 4days	5	430 ±60	505 ±55	664 ±82	772 ±104	768 ±96	723 ±75
6days	5	376 ±104	470 ±144	587 ±150	725 ±179	670 ±169	660 ±175
10days	11	436 ±50	500 ±47	644 ±52	751 ±59	732 ±58	657 ±57

4.2 BEHAVIORAL AND PHYSIOLOGICAL

4.2.1 Methods

The wealth of different behavior patterns from which to choose those to be measured in any specific study presents problems of selection. Two approaches to selection have received most attention. One involves an a priori classification of behavior, sometimes accepting results of factor analyses of a wide variety of behaviors as the starting point and selecting behaviors representative of one or more factors to be included in a test battery. A second approach begins with some systematic model of behavior. In the present instance we have used a general model derived form communication theory which analyzes behavior in terms of such processes as sensory input, central fixation and storage, central retrieval or readout and motor output. The assay techniques selected on this basis are described below. All are well known in behavioral research, although the details of their applications have been modified to meet our specific needs.

(1) Body weight

Body weights were recorded daily during the course of the experiments using a Sartorius High Capacity Balance Model 1404 MP8 and a Sartorius Model 7279 printer. These measures served as indices of general health and a measure of possible effects of the BM 123 regimen on caloric intake.

(2) Core body temperature

Core body temperature was measured using a YSI Model 49 TA digital thermometer, with an accuracy within the range of tempertures recorded of ±0.05°C. YSI series 500 probes fed information into the thermometer. The probes were of the thermistor type with fast response times. They were small in physical size, facilitating insertion into the rectum to a depth of 6 cm. Baseline temperatures were taken prior to treatment and were recorded at the various time intervals coinciding with the measurement of cholinomimetic signs.

(3) Cholinomimetic signs

The occurrence or non-occurrence of three general signs of muscarinic activity, i.e., tremor, salivation, and chromodaccyhrea, was recorded at various intervals starting immediately after each experimental treatment. The selection of particular times depended on the specific compound being tested (i.e., in some cases, signs were recorded immediately after the injection and monitered at 5 min intervals; in other case, signs were monitored at 15 min of 30 min). The times selected were based upon observations during preliminary experiments. In most cases, measurements were recorded up to and including 3 h after injection.

(4) Body fluid balance

On arrival in the laboratory, animals were placed in individual home cages where they soon acquired the simple operant response of drinking water from metal drinking tubes. The tubes were connected to graduated cyclinders attached to the front of the cages. Water intake was recorded in ml during 20 min drinking periods when animals were on water deprivation schedules, otherwise at 24 h intervals.

(5) General activity

Three traditional techniques for measuring general activity are available and have been used in earlier stuides. Two are "observational techniques", i.e., the open field and the more coplex environment of the Y-maze employed extensively in evaluating psychoactive agents. The third technique, a tilt cage, provides for automatic recording. For our purposes, we decided to adopt the open field technique for computer recording and anylsis. General activity of the rats was measured in circular open field chambers with a diameter of 60 cm. The interior walls of the chambers were fitted with two sets of infrared-sensitive photocells and light emitting diodes. One set, 4 cm above the floor, measured horizontal (locomotor) activity, while the second set, located 12 cm from the floor measured vertical (rearing) activity. The floor was composed of a fine screen wire mesh, and the chambers were covered with plywood tops. Red light (50 watt bulb) illumination and white noise were used to maintain constant conditions of visual and auditory stimulation. The activity chambers were interfaced with a TRS-80 Model III microcomputer which automatically recorded all light beam breaks and, at the end of each animal's daily session, printed the results in terms of horizontal and vertical activity during each 2 min interval. Activity was defined as the total beam breaks during a daily session.

(6) Habituation

By sampling activity as a function of time in the open field situation it was possible to obtain measures of habituation, a type of non-associative learning (78). Habituation has been defined as the most ubiquitous form of learning. Non-associative modesl of behavioral changes induced by central cholinergic drugs have been considered as more pragmatic than associative models in understanding many research results. Its relation to changes in transmitter realse, receptor senstivity and second messanger systems have been explored. Within the present context "habituation" is used as a theoretical construct, while its observable correlates are operationally measurable in terms of decrements in behavioral responding. The sampling procedure referred to above provided this basic measure of acquisition by recording activity at regular intervals of 2 min for a period of 20 min. In control animals plots of activity at each for these intervals produce hyperbolic curves characteristic of habituation. ANOVA for repeated measures within a particular

treatment group provides a test of whether the systematic adaption represented by such curves is statistically signigicant. Curve fitting procedures may be used to generate parameters that enable compromises to be made between treatment groups.

(7) Nociception (algesia)

Reviews of the research literature suggest that at least in rodents, and probably also in humans, administration of cholinergic agonists is associated with consequent analgesic effects. Our earlier experience with techniques for studying nociception, i.e., the tradition "hot plate" and "tail flick" approaches led us to seek a more satisfactory and more precise method. This resulted, early in the period of the present contract, in the development of what we have called the "up-and-down" procedure (79).

Measurement of footshock, i.e. flinch and jump, thresholds involved placing the animal in a test chamber, the floor of which consisted of stainless steel rods through which electric shocks of varying intensity could be delivered. Shock intensities were available from 0.02 ro 2.0 mA in 20 steps arranged logarithmically. Use of the full range of intensities was never necessary in determining thresholds. Each shock pulse (60 Hz) had a duration of 0.5 sec and shocks were delivered at 10 sec intervals. Shock levels at the start of an up-and-down series were set at midpoints of the ranges within which preliminary experiments had shown the thresholds likely to occur. The experimenter then adjusted the intensity in accordance with the animal's response on each particular trial, i.e. raised 0.1 log unit when no response occurred and lowered 0.1 log unit when a reponse had been made. A "flinch" was defined as elevation of one paw from the grid floor and "jump" as rapid movememnt of three or four paws involving withdrawal from the floor. As shock intensities increased from zero, initial responses (flinch) were primarily sensory reflexive, followed by behaviors involving both sensory and perceptual processes (80). Thresholds were measured daily for 4 days and subsequently 2 times weekly.

(8) Conditioned avoidance response (CAR)

The discrete trial CAR has seen wide use as both a measure of learning (acquisition) under conditions of aversive reinforcement and of memory. It has been used as a "one way" CAR in both capacities during the present experiments. The apparatus used in studying CAR consists of the traditional straight alley with compartments at each end (81). In our apparatus, the sides of the alley are molded from two sheets of stainless steel to provide a U-shaped trough, separated at the bottom and divided into three sections. An electric shock control unit can be set to deliver a pre-determined current flow to any of the sections. An auditory signal is provided to indicate the start of a trial (one-way) problem or a more complicated alternation (two-way task). This assay requires locomotor (spatial) responses during learning and performance of the learned response. Several

measures of behavior are provided: motor capacity, learning, memory and transfer of training from one pharmacological state to another.

(9) Fixed rate operant response (FR5)

FR5 responding was included in the battery of assays in order to assess effects of the various treatments on a relatively simple behavior pattern which is dependent upon temporal rather than spatial cues. Five operant chambers provided the subject environments. Each was equipped with a lever, a drinking trough into which 80 µl of water were delivered when the required number of presses occured, a house light, and a stimulus light. The chambers were positioned in sound-atenuated boxes to exclude interference by extraneous auditory or visual stimuli. A TRS-80 Model III microcomputer, interfaced with the chambers, was used both for programming the operant schedule and for recording the subject's responses. A bypass unit enabled the experimenter to control water delivery during the shaping of naive subjects to lever press. Animals involved in these experiments were maintained on a 23.5-h daily water deprivation schedule, trials for FR5 responding being carried out during the remaining 30 min.

(10) Inhibited (passive) avoidance

The inhibited (passive) avoidance paradigm has been used frequently to provide a measure of memory (78). A 1-trial step-through inhibitory avoidance task was used in the present experiments. A trough-shaped alley constructed of stainless steel was divided by a pivoted door into a lighted safe compartment and a dark shock compartment, the door was closed and an inescapable footshock (1.0 mA, 0.5 sec) was delivered.

After delivery of the shock, the rat was removed from the alley and returned to its home cage. The test for retention was given 24 h later, using the same procedure as on the learing trial, but with the shock omitted. Times taken by the animal to enter the dark compartment on trials 1 and 2 were measured. If the response had not been made within 600 sec, the trial was ended and the animal was assigned a score of 600.

4.2.2 Results

(1) Median lethal dose (LD_{50})

LD_{50}s were determined for both BM130 and BM130A IV using the up-and-down method. Experiments were carried out in duplicate for mice and in quadruplicate for rats. There was a pronounced interspecies difference in LD_{50} for BM130, that for mice (9.6 µmol/kg) being approximately twice that for rats (4.2 µmol/kg). Median lethal doses for BM130A in mice and rats were 1.6 µmol/kg and 0.90 µmol/kg, respectively. The standard error of the estimate corresponds to a factor of 1.14 for all LD_{50} determinations.

(2) General signs: Preliminary observations

An initial series of observations using the sign checklist and involving 10 naive rats provided broad guidelines within which to plan more detailed studies. BM130 was administered IV at dose levels of 0.1, 0.5, 1.0, 1.5 and 3.0 μmol/kg. There were no noticeable effects of injections of saline or of 0.1 μmol/kg BM130. Above the latter dose level, tremor, indicative of central action, occurred with dose-dependent intensity. Signs of peripheral effects, e.g. chromodacryorrhea and salivation, showed dose dependencies at the 1.0 μmol/kg level and above, but were not noted at 0.5 μmol/kg. Preening occurred within this range, but only after a time lag which suggested that it was a consequence of salivation. No other effects were seen. Both central and peripheral effects appeared within 1 min of injection, the former being of shorter duration than the latter.

Two and a half hours after their initial treatment these animals were challenged with oxotremorine (1.0 μmol/kg). Tremor was noted during the first 5 min after injection in all subjects. The intensity of the tremor was inversely dependent on the prior dose of BM 130, i.e., only slight for the higher BM 130 dose animals and more pronounced in those that had received 0.1 μmol/kg BM 130 or saline. Peripheral signs (chromodacryorrhea, salivation, diarrhea) were also inversely related to initial dosages of BM 130.

Effects of repeated administrations of BM 130 at doses of 1.5 and 3.0 μmol/kg provided further preliminary information. Four injections were given IV at 15 min intervals to two naive rats at each dose level. Tremor was pronounced after the first administration and was not observed thereafter, suggesting rapid development of tolerance for the central effects of BM 130. By contrast peripheral signs, i.e., chromodacryorrhea, salivation, and diarrhea, persisted throughout three or four of the injection series, indicating that tolerance developed more slowly in peripheral mechanisms. An oxotremorine challenge (1 μmol/kg SC.) at the end of the series produced slight tremor and clearly observable peripheral signs, which persisted for 15 min or more.

In order to obtain some preliminary information about the possible duration of the effects of acute administration two animals that had received BM 130 and one saline 6 days earlier were challenged with oxotremorine (1.5 μmol/kg SC). Slight tremor was seen in one of the former two; peripheral signs (chromodacryorrhea, salivation, diarrhea) were present in both. Tremor and the peripheral signs appeared in the animal treated earlier with saline.

(3) Acute administration

On the basis of these preliminary findings, three types of experiments were carried out. The first was designed to study in detail central and peripheral signs after acute administration of

various doses of BM 130 and also of a standard dose of BM 130A, contrasting each with a saline control group. Observations described above supported the decision to focus on three effects: tremor as an indicator of central actions, and chromodacryorrhea and salivation, of peripheral involvement. Results from observations of 71 rats are summarized in Table 5, expressed as percents of animals affected. It is quite clear that injections of BM 130 within the dose range, 1.5 to 3.0 μmol/kg, produced both central and peripheral effects. BM 130A treated subjects showed peripheral, but not central signs and saline produced none of them. Fisher's Exact Test confirmed that differences between the BM 130 and saline animals were significant at $p < 0.01$.

(4) Duration of effects

Further experiments were carried out to obtain information about the possible long term actions of BM 130 and BM 130A on the cholinergic system after disappearance of the acute effects. Tests were made using the cholinergic agonist, oxotremorine, at a standard dose level of 1.0 μmol/kg s.c. Challenges were made at weekly intervals beginning 7 days after acute injections and continuing for three weeks. Results are summarized in Table 6. Certain trends in the data deserve special attention. During the first two weeks animals receiving the highest dose of BM 130 were more resistant to the central effects of oxotremorine than those administered lower doses: chi square=3.28, $p < 0.05$ in both cases. Secondly, no such resistance appeared in the peripheral signs during any of the challenges and was not noted following pretreatment with BM 130A or saline. In the third week there was a small decrease in central effects for all pretreatments including saline, suggesting adaptation to repeated injections of oxotremorine.

(5) Effects of repeated administrations

Effects of repeated administration of BM 130 were studied in an experiment in which groups of five rats received Saline, BM 130, 2.4 μmol/kg, or BM 130A, 0.3 μmol/kg, at 15-min intervals. Observations were made using the primary signs: tremor, chromodacryorrhea, salivation. Results are summarized in Table 7. Following the first two injections all animals in the BM 130 group showed tremor, but none in the BM 130A or saline groups. All BM 130 and BM 130A rats had signs of peripheral involvement and none in the saline group. Mean durations of the BM 130 effects decreased from the first to the second administrations and by the third injection, i.e. 30 min after start of the series, no signs of central or peripheral involvement were observable. The dose of BM 130A administered produced peripheral signs only and these disappeared by the fourth treatment. None of the various signs were seen in the saline animals.

TABLE 5

GENERAL SIGNS: ACUTE ADMINISTRATION

Percent of Animals Affected

TREATMENT (μmol/kg)		n	SIGN (% AFFECTED)		
			CENTRAL:	PERIPHERAL:	
			Tremor	CHROMO-DACRYORRHEA	SALIVATION
BM130	(3.0)	12	100	100	100
BM130	(2.4)	9	89	100	75
BM130	(1.9)	10	80	100	90
BM130	(1.5)	5	100	100	100
BM130A	(0.3)	17	0	88	47
SALINE		18	0	0	0
		N=71			

TABLE 6

GENERAL SIGNS:
RESISTANCE TO OXOTREMORINE CHALLENGES FOLLOWING ACUTE TREATMENTS
Percent of Animals Affected

TREATMENT (µmol/kg)		n	CHALLENGE Week	SIGN (% AFFECTED) CENTRAL Tremor	PERIPHERAL CHROMO-DACRYORRHEA	PERIPHERAL SALIVATION
BM130	(3.0)	12	1	58	100	100
			2	67	100	100
			3	67	92	100
BM130	(2.4)	5	1	100	100	100
			2	100	100	100
			3	60	100	100
BM130	(1.9)	5	1	100	80	100
			2	100	100	100
			3	40	100	100
BM130A	(0.3)	12	1	92	92	83
			2	92	100	100
			3	75	100	100
SALINE		14	1	93	93	100
			2	93	100	100
			3	71	100	100
		N=48				

TABLE 7

GENERAL SIGNS: EFFECTS OF REPEATED ADMINISTRATIONS
Percent of Animals Affected

TREATMENT (µmol/kg)	n	INJECTION	SIGN					
			TREMOR		CHROMODACRYORRHEA		SALIVATION	
			%	Mean duration (min)	%	Mean duration (min)	%	Mean duration (min)
BM130 (2.4)	5	1	100	8.0	100	15.0	100	14.0
		2	100	6.0	40	7.5	40	10.0
		3	0	-	0	-	0	-
		4	0	-	0	-	0	-
BM130A (0.3)	5	1	0	-	80	8.8	0	-
		2	0	-	20	5.0	0	-
		3	0	-	20	10.0	20	5.0
		4	0	-	0	-	0	-
SALINE	5	1	0	-	0	-	0	-
		2	0	-	0	-	0	-
		3	0	-	0	-	0	-
		4	0	-	0	-	0	-
		N=15						

(6) Body weight

Body weights were recorded during a period of 3 weeks following acute administration of BM 130, BM 130A or saline. There was the expected consistent and significant trend toward increasing weight as the animals grew older ($F_{(3,63)}=163.57$, $p < 0.01$). On Day 22 there were no significant differences among groups despite the different pharmacological treatments they had received ($F_{(2,21)}=0.21$, $p > 0.05$). Clearly there were no signs of inadequate caloric intake nor confounding effects of poor physical condition between the groups.

(7) Core body temperature

Two experiments were carried out to study effects of the compounds on core body temperature. The first was designed to obtain information about changes in temperature over time after injection and the second, to define dose-effect relations. Doses for the former were selected on the basis of results reported above, i.e., BM 130, 3.0 and BM 130A, 0.3 μmol/kg. ANOVA for repeated measures during the 4 days preceding drug treatments established that there were no significant differences among the drug and saline control groups: $F_{(2,21)}=0.28$, $p > 0.05$.

Two-way ANOVA of data from the first experiments showed significant differences in effects of the three treatments during a period of 4 h immediately after their administration: $F_{(2,21)}=5.09$, $p < 0.02$. It also indicated that the differences did not remain constant as time after treatment increased: for repeated measures, $F_{(3,63)}=18.97$, $p < 0.01$. Graphic inspection of the data suggested that the significant differences occurred at 30 min after injection, temperatures returning to preinjection levels thereafter. This was substantiated by ANOVA, which at 30 min gave $F_{(2,21)}=20.66$, $p < 0.01$ and at 60 min, $F_{(2,21)} = 1.78$, $p > 0.05$. Scheffe comparisons showed that both drug treatments differed from the saline control and from each other: BM 130 induced hypothermia and BM 130A, hyperthermia.

ANOVA for body temperatures at the peak effect time of 30 min (Table 8) showed a significant difference among the five groups in the second experiment: $F_{(4,18)}=8.03$, $p < 0.01$. Scheffe analyses were carried out comparing the various treatment groups with the saline group as the control. From the results it was clear that BM 130 animals were significantly hypothermic at the highest dose, with apparent dose related effects at lower doses. The highest dose also produced significant hypothermia when compared with effects of BM 130A, which were not different in this experiment from those of the saline treatment. Comparisons of the BM 130A and of the Saline groups from the two experiments showed that there was no significant difference between the former ($t_{10}=1.75$, $p > 0.05$), but that Saline animals had a significantly higher mean temperature in experiment 2 ($t_{13}=2.19$, $p < 0.05$). This accounted for the fact that BM 130A animals were relatively hyperthermic in the first experiment and not in the second.

TABLE 8

Effects of BM130 and BM130A on Core Body Temperature: Mean (SEM)

Treatment (μmol/kg)	Body Temperature (°C)	
	Pretreatment	30 min after injection
BM130 (2.4)	38.4 (0.43)	37.2 (0.29)
BM130 (1.9)	38.8 (0.34)	38.0 (0.35)
BM130 (1.5)	38.3 (0.87)	38.3 (0.29)
BM130A (0.3)	38.6 (0.44)	39.2 (0.17)
SALINE	38.5 (0.51)	38.8 (0.08)

All groups, including the saline control subjects, showed hypothermic reactions to oxotremorine challenges, the mean decrease being 1.6±0.10°C. However, these effects did not differ significantly among the various BM 130 and BM 130A groups during any of three challenges: 1, $F_{(2,21)}=0.12$; 2, $F_{(2,21)}=0.58$; and, 3, $F_{(2,21)}=0.05$; in all cases $p > 0.05$.

(8) Body Fluid Balance (Water Intake)

Data on 24-hr water consumption were obtained for 5 groups of animals during acute treatment with BM 130 (3.0, 2.4, 1.9 μmol/kg), BM 130A (0.3 μmol/kg) or saline. ANOVAs for measures of intake during the 24 h preceding administration, the 24 h after injection and the next 24 h post-treatment period showed no significant between group differences: $F_{(4,19)}=0.26$; $F_{(4,19)}=0.30$; and $F_{(4,19)}=1.47$, respectively.

Analyses were also carried out of water intake during each of three subsequent weekly oxotremorine challenges (1.0 μmol/kg) spaced at weekly intervals. Again there were no significant differences between the various treatment groups on any of the challenge days: $F_{(4,19)}=2.48$; $F_{(4,19)}=1.77$; $F_{(4,19)}=1.10$, respectively.

(9) Nociception (Algesia)

ANOVA of pretreatment measures showed no significant differences between groups for flinch or for jump thresholds: $F_{(2,21)}=1.75$ and $F_{(2,21)}=1.03$, respectively (see Table 9). 2-way ANOVA for differences between these pretreatment thresholds and thresholds after acute injections, i.e. Flinch and Jump, indicated that there were significant differences between the BM 130, BM 130A and Saline groups for both variables: Flinch, $F_{(2,21)}=4.05$, $p < 0.05$; Jump $F_{(2,21)}=4.22$, $p < 0.05$. Within group differences, i.e. at 30, 60, 120 and 240 min after acute injection, were not significant: Flinch, $F_{(3,63)}=2.06$; Jump, $F_{(3,63)}=0.77$. Treatment x time interactions were not significant for either flinch or jump. Further analyses showed that the significant difference between groups was attributable to the fact that BM 130A animals were consistently hyperalgesic, i.e. had lower flinch and jump thresholds, throughout the 4 hr of measurements after injection.

Results of the oxotremorine challenges are summarized in Table 9. ANOVA showed that effects of the first weekly challenge were to cause hypoalgesia (elevated thresholds) for both flinch and jump measurements $F_{(2,21)}=7.95$, $p < 0.01$; $F_{(2,21)}=4.24$, $p < 0.05$), respectively. Scheffe comparisons established that the effect had occurred in the saline control and BM 130A animals, those receiving BM 130 being not significantly different from their pretreatment thresholds. The differences had disappeared by the end of the second week (challenge 2) for flinch thresholds ($F_{(2,21)}=1.46$), but at the same time were still evidenced in jump thresholds ($F_{(2,21)}=3.93$, $p < 0.05$). There were no significant differences between the

TABLE 9

NOCICEPTIVE THRESHOLDS (mA) 30 MIN FOLLOWING OXOTREMORINE CHALLENGES

Threshold	Baseline	Oxotremorine Challenge		
		1 Mean±SEM	2 Mean±SEM	3 Mean±SEM
FLINCH				
BM130	0.18±.027	0.17±.026	0.19±.032	0.21±.026
BM130A	0.17±.021	0.24±.033	0.18±.026	0.22±.017
SALINE	0.13±.015	0.19±.019	0.21±.014	0.18±.015
JUMP				
BM130	0.35±.030	0.37±.046	0.41±.064	0.54±.056
BM130A	0.37±.035	0.51±.045	0.43±.054	0.51±.015
SALINE	0.30±.024	0.50±.058	0.54±.037	0.52±.038

groups by the end of the third week ($F_{(2,21)}=0.19$; $F_{(2,21)}=0.94$). Within group differences in the duration of effects at 30, 60, 120 and 240 min after injection were highly significant: Flinch, $F_{(3,63)}=11.33, p < 0.01$; Jump, $F_{(3,63)}=21.38$, $p < 0.01$. Scheffe comparisons indicated that BM 130A and Saline animals were affected initially, but recovered by the time of the 2 hr assay. BM 130 animals were resistant throughout this period.

(10) Conditioned Avoidance Response (CAR)

Preliminary training had ensured that all animals had reached the criterion of 7 CARs in 10 trials before experimental treatments began. Acute administration of the various treatments showed no significant effects on trials to meet the criterion of 7 in 10 Ra responses 30 min after injections ($F_{(4,19)}=1.19$, $p > 0.05$), the median number of trials for the two high doses of BM 130 being 1 and for the other group, 0. There were no instances of time out, no significant differences in times to make avoidance responses ($F_{(4,19)}=0.26$, $p > 0.05$), and too few escape responses to warrant statistical analysis.

Two-way ANOVAs of data produced by the three weekly oxotremorine challenges (Table 10) which followed acute injections also revealed no treatment effects: time out, $F_{(4,19)}=0.68$; trials to criterion, $F_{(4,19)}=0.60$; mean Ra times, $F_{(4,19)}=2.15$; in all cases $p > 0.05$. However, there were general within group trends toward decreasing magnitudes of effect with successive challenges: time out, $F_{(2,48)}=8.77$, $p < 0.01$; trials to criterion, $F_{(2,48)}=10.58$, $p < 0.01$; mean Ra times, $F_{(2,48)}=2.98$, $p < 0.05$. Such trends were evident in all groups for the three challenges with the exception of the behavior of the BM 130A animals, which showed no consistent changes in any of the behaviors during the challenge series.

(11) Fixed Ratio Operant Behavior (FR5)

There were no significant differences among the various treatment groups in predrug baseline performance of the FR5 behavior: $F_{(4,18)}=2.45$, $p > 0.05$. As with other measures of learned behaviors reported above, no significant differences between groups were found when FR5 responding was assayed 30 min after acute administration of the three doses of BM 130, of BM 130A or of Saline: $F_{(4,18)}=1.95$, $p > 0.05$. However, there were significant differences in recovery when FR5 performance (Table 11) was measured 24 h after the injections: $F_{(4,18)}=5.39$, $p < 0.01$. Scheffe comparisons showed that the significance was due primarily to the rapid recovery of the high dose group, which had returned to the level of saline control responding within the 24 h. Performance of the BM 130A animals did not differ from that of saline controls.

ANOVAs for treatment effects in FR5 responding after the first and second weekly oxotremorine challenges also failed to be significant: $F_{(4,18)}=2.40$ and $F_{(4,18)}=2.73$, respectively; $p > 0.05$ in both cases. Observation of animals performing during

TABLE 10

Effects of Oxotremorine Challenges On
Parameters of the Conditional Avoidance Response: Mean (SEM)

Parameter	Treatment (μmol/kg) BM130(3.0)	BM130(2.4)	BM130(1.9)	BM130A(0.3)	Saline
A. Trials to criterion					
Challenge					
1	12.0(5.6)	10.4(4.1)	15.8(6.8)	15.8(6.1)	14.0(4.9)
2	9.4(6.1)	9.0(5.1)	7.0(3.3)	13.8(4.9)	9.5(2.7)
3	3.6(2.0)	0 --	6.8(2.4)	12.0(2.1)	7.8(1.0)
B. "Time Out" (min)					
1	50.8(19.9)	45.0(16.6)	25.4(17.1)	33.8(14.7)	33.0(10.2)
2	23.0(14.6)	18.6(8.2)	17.0(6.0)	20.0(4.7)	8.8(6.4)
3	9.0(6.8)	0 --	9.0(5.3)	31.0(4.0)	12.5(2.9)
C. Avoidance response (R_a)					
1	7.0(2.0)	6.1(1.0)	5.8(1.8)	5.5(1.6)	6.2(1.1)
2	6.4(0.7)	4.0(0.6)	4.9(1.7)	6.0(1.3)	5.3(1.2)
3	4.9(1.6)	3.4(1.1)	5.6(2.1)	5.7(2.5)	6.4(1.4)

TABLE 11

Recovery of FR_5 Operant Behavior[1]
Following (24h) Acute Injections of BM130 and BM130A: Mean (SEM)

Treatment (μmol/kg)	Pretreatment	Acute	Recovery[2] OX01	OX02	OX03	OX04
BM130(2.4)	149.5 (17.43)	172.8 (18.84)	203.3 (16.02)	213.0 (14.43)	162.5 (16.30)	155.5 (12.71)
BM130(1.9)	139.6 (20.67)	129.6 (28.05)	138.2 (33.08)	177.4 (21.36)	142.0 (23.81)	143.2 (25.31)
BM130(1.5)	196.2 (16.52)	164.8 (18.90)	231.6 (20.87)	229.8 (27.29)	178.4 (25.32)	137.4 (14.55)
BM130A(0.3)	142.6 (12.75)	128.8 (10.86)	174.8 (15.72)	176.4 (10.73)	139.8 (15.35)	148.2 (12.54)
SALINE	179.3 (10.16)	184.3 (12.91)	262.3 (9.68)	262.5 (13.87)	212.5 (12.04)	161.5 (15.82)

[1]Number of responses (reinforcements) in 30 min.
[2]24 h after acute injection.

the experimental period and later examination of measures taken showed that these results were due to the suppressing effects of the dose of oxotremorine injected, i.e., 1.0 μmol/kg, which reduced responding in all groups essentially to zero during the assay period (Table 12). That, in fact, there were carryover effects of the initial acute injections became evident in the third and fourth weekly challenges when the dose of oxotremorine was reduced to 0.25 μmol/kg. ANOVAs of data from these two challenges provided $F_{(4,18)}=6.17$, $p < 0.01$ and $F_{(4,18)}=3.69$, $p < 0.02$, respectively. Scheffe comparisons showed that the significant effects were related to dose dependent trends among groups treated with BM 130. During both these challenges animals pretreated with the high dose of BM 130 (2.4 μmol/kg) were less affected by oxotremorine than those receiving the next highest dose (1.9 μmol/kg) and both less than those that had received the lowest dose (1.5).

4.2.3 Effects of oxotremorine and its mustard, BM 123

A second series of experiments is still in progress. It was designed to study effects on behavioral and physiological variables of another alkylating analog of oxotremorine, BM 123, using an injection regimen by which mAChRs are downregulated to less than 20% of normal. Oxotremorine, administered using a similar regimen, was included for purposes of comparison. Our early analyses of the results may be summarized briefly as follows.

BM 123 was injected into the tail vein of Sprague-Dawley rats in doses of 8, 20 and 50 mol kg^{-1} at 1 hr intervals. This procedure reduces the mAChR to 10% of normal as judged by [^{3}H-]QNB binding. In other experiments oxotremorine was injected in a similar series of doses (0.3, 0.75, 1.88 mol kg^{-1}). Measurements of behavioral and physiological variables began immediately after completion of the injections and continued daily for 26 days.

The time course of the changes induced by BM 123 varied widely. Some variables (e.g. tremor, chromodacryorrhea) showed peak changes in 5 min. and returned to their pretreatment baselines within 5-30 min.; body temperature and nociceptive thresholds showed peak changes of -2.6 °C and + 270% and returned to normal within 6 and 24 hr respectively. Locomotor activity and learned operant responding were impaired for 4 and 8 days. After return to baseline some variables showed a significant rebound in the opposite direction. Only the performance of the fixed interval operant response paralleled the return of the mAChR to their normal levels. All changes elicited by oxotremorine recovered more rapidly than those produced by BM 123, confirming that the latter produces a sustained change in receptor-mediated events which would be expected from an irreversible ligand. These results suggest widely differing sensitivity of different neural circuits to inteference by BM 123. The differences could be due to the stabilizing effect

TABLE 12

Effects of BM130, BM130A and of Oxotremorine Challenges on FR_5 Operant Behavior[1]: Mean (SEM)

Treatment Challenge[2,3] (μmol/kg)	Pretreatment Baseline	Acute Injection	Oxotremorine 3	4
BM130(2.4)	149.5 (17.43)	137.5 (31.76)	112.8 (26.91)	100.8 (15.97)
BM130(1.9)	139.6 (20.67)	112.0 (24.06)	95.4 (14.17)	75.2 (6.22)
BM130(1.5)	196.2 (16.52)	156.4 (20.30)	82.8 (12.73)	86.0 (19.03)
BM130A(0.3)	142.6 (12.75)	109.4 (15.90)	76.0 (12.74)	92.0 (16.11)
SALINE	179.25 (10.16)	176.3 (16.85)	97.0 (9.35)	75.8 (11.20)

[1] Number of responses (reinforcements) in 30 min.
[2] Challenges 1 and 2 were at doses of 1.0 μmol/kg; 3 and 4, at 0.25 μmol/kg.
[3] There were too few responses to justify statistical analyses for challenges 1 and 2.

of neural feedback loops or to the presence of a large receptor reserve at some sites.

4.2.4 Withdrawal following chronic DFP treatment

Although there is considerable information about the development of tolerance during chronic administration of DFP, few comments about effects of abrupt withdrawal have appeared in the research literature. An early report showed withdrawal to be associated with a supranormal level of relatively long duration in the performance of a simple operant response. One of the major objectives of the present research program is to investigate withdrawal effects in greater detail. Such a program is now underway. Data are still being collected and only early results can be reported here.

The first experiment in this series was designed to measure several behavioral and physiological variables at selected times during a 29 day period following 10 days of chronic DFP treatment. All injections were subcutaneous in a standard volume of 1.0 ml/kg body weight. The regimen for DFP subjects was selected after preliminary tests and were similar to those used previously in our laboratory. On the first treatment day experimental animals received injections of 2.0 mg/kg DFP. After a 72 hr period of no treatments chronic subjects were administered 0.5 mg/kg DFP daily until their full compliment was reached in a total of 10 days. This duration of chronic treatment was chosen on the basis of earlier reports that by 10 days tolerance has developed as indicated by the disappearance of parasympathomimetic signs and return to pretreatment baselines of a wide variety of behaviors. Paired control animals were given arachis ail, the vehicle for DFP, on an identical schedule. The experiment was replicated to provide information about the consistency of results.

The considerable amount of data are still being analyzed by 1- and 2-way ANOVAs (the latter involving repeated measures), followed by post-hoc comparisons using the Scheffe method. Some very preliminary results suggest that nociceptive thresholds were elevated significantly throughout the 10 days of chonic DFP treatment, i.e. tolerance did not develop. This effect is characteristic of cholinergic agonists and is consistent with the elevation of ACh levels following decreases in AChE activity. Recovery to control levels occurred in approximately 5 days following abrupt withdrawal of the DFP regimen. By contrast, both parameters of the activity assay, horizontal and vertical movement, did evidence tolerance during chronic treatment. The horizontal component adapted somewhat sooner than the vertical, i.e. 6 vs 8 days. There were no significant differences between DFP and control subjects following abrupt withdrawal. Such results support the general conclusion reached in much earlier research: DFP produces differential effects in behavior, evidenced by the fact that some behaviors are affected and others not. This and other statements about withdrawal after chronic

DFP treatment depend upon completion of the analyses of the experimental data now underway.

This series of experiments includes assays for whole brain AChE activity levels and for the release of ACh from the myenteric plexus preparation. Both were assayed 24 hr after the 10 days of chronic administration and in other groups of animals at 8, 15 and 29 days following abrupt withdrawal of DFP. At 8 days stimulated release of ACh from the myenteric plexus (taken in relation to resting release) approximated control levels, where it remained when measured at 15 and 29 days. By comparison, AChE activity levels were approximately by 50% of control values at 8 days, reaching control levels at 15 and 29 days.

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5. DISCUSSION AND CONCLUSIONS

The following paragraphs discuss conclusions drawn from the results of the various experiments reported above.

5.1 Neurochemical and Pharmacological

5.1.1 Basic properties of oxotremorine analogs

BM 123 and BM 130 cyclize in aqueuos solution at neutral pH to form aziridinium ions, concurrently liberating one equivalent of chloride ion. The kinetics of the formation of the aridinium ion differed markedly for the two compounds. Chloride ion was release extremely slowly from BM 123. The rate constant for the cyclization of BM 123, as measured by chloride release, was lower than the rate constant for the decay of aziridinium ion I. Furthermore, at 37 °C only about 50% of the theoretical amount of chloride ion had been released from BM 123 at the time of maximum aziridinium ion concentration. These observations satisfactorily explain the relatively low conversion of BM 123 to I and suggest no need to invoke competing reactions, e.g. reaction of BM 123 with the aziridinium ion to form a piperazinium dimer. In contrast, chloride ion was released rapidly from BM 130 leading to a relatively higher peak concentration of the aziridinium ion II.

A number of observations suggest that the aziridinium ions I and II are the molecular species mainly responsible for the muscarinic actions of BM 123 and BM 130. For example, the in vitro muscarinic activity of solutions of BM 123 and BM 130 at different stages in the cycle of generation and hydrolysis of the aziridinium ions closely paralleled the aziridinium ion concentrations as determined by thiosulfate consumption. Thiosulfate treatment of a solution of BM 123, which showed maximal muscarinic activity, almost completely abolished the activity. Since this solution contained about 60% of the original amount of BM 123, and since thiosulfate does not react directly with 2-chloroethylamines, the disappearance of the muscarinic activity after treatment with thiosulfate not only points to the aziridinium ion as the active species, but also suggests that BM 123 has very weak if any muscarinic activity. Similar treatment of a solution of BM 130 completely abolished the muscarinic activity. However, this observation does not rule out possible muscarinic activity of BM 130. Since thiosulfate added to the organ bath did not protect against the muscarinic actions of BM 130, it appears that the interaction of the aziridinium ion II with muscarinic receptors proceeds more rapidly than its reaction with thiosulfate. Considering the rapid cyclization of BM 130, it is therefore difficult to prove unequivocally the inactivity of the parent 2-chloroalkylamine. However, from structure-activity relationships in other analogues of oxotremorine, including the 2-methylpyrrolidino (82) and the 2-hydroxymethylpyrrolidino (this work) derivatives, one would expect BM 130 to have antagonistic rather than agonistic properties. Moreover, BM 130 (pK_a 6.37) is largely unprotonated

at physiological pH and it is known that the muscarinic activity of tertiary amines is associated with their protonated forms (83). Finally, the alcohol formed by hydrolysis of BM 123, i.e. III, is a very weak muscarinic agent whereas the alcohols derived from BM 130, i.e. IV and V, are weak muscarinic antagonists.

BM 123 was only slightly less potent than oxotremorine-M (Table 1) and equipotent with oxotremorine in the guinea pig ileum. If one takes into account the poor yield of the aziridinium ion I from BM 123, then I should be more potent than oxotremorine-M. BM 130 was 3-4 times more potent than oxotremorine methiodide. It therefore appears that the minor structural modification of connecting two methyl groups of oxotremorine-M or of connecting the methyl group with the alpha-carbon of the pyrrolidine ring of oxotremorine methiodide increases muscarinic activity. At nicotinic receptors of the frog rectus abdominis muscle, this structural modification reduces the activity of oxotremorine-M but has no effect on the activity of oxotremorine methiodide. The selectivity of these compounds for muscarinic as opposed to nicotinic receptors may be expressed as the ratio of the ED_{50} values for contraction of the frog rectus and of the guinea pig ileum. BM 123 has the highest selectivity, being about 175-fold more selective for muscarinic receptors than carbachol. The low nicotinic activity of BM 123 is in sharp contrast to the profound nicotinic effects of acetylcholine mustard (84).

In homogenates of the guinea pig ileum, BM 123 and BM 130 caused a selective reduction in the binding capacity of [^{3}H]NMS without significantly affecting the apparent affinity. This inhibitory effect persisted after extensive washing, indicating a covalent interaction of the aziridinium ions of BM 123 and BM 130 with ileal muscarinic receptors. These results and the high potency of the aziridinium ions in stimulating contractions of the ileum, suggested that, with higher concentrations and/or prolonged exposure, alkylation of receptors could occur in whole ileum leading to a persistent functional blockade. An often used measure of drug antagonism is the agonist dose ratio, i.e. the ratio by which the agonist concentration must be increased in order to produce a standard response after the addition of the antagonist. The fraction of receptors occupied by the antagonist may be calculated from the agonist dose ratio. There are, however, experimental difficulties involved in using agonist dose ratios calculated from contractile responses as a measure of irreversible binding of a stimulant drug. Prolonged exposure to high bath concentrations of such a drug will cause receptor desensitization which initially may be indistinguishable from irreversible binding. Also, the relationship is valid only if the agonist used to measure dose ratios occupies a negligible fraction of the total amount of receptors available. In the present study, the time required for recovery from desensitization caused by oxotremorine-M was used an approximate measure of the recovery from desensitization after exposure to BM 123 and BM 130. Oxotremorine-M was also the agonist used to

measure dose ratios since it has a very large spare receptor capacity in the guinea pig ileum. With this method, BM 123 and BM 130 (20 μM for 30 min) were found to occupy permanently 94% and 85%, respectively, of the receptors. Strong support for the validity of these calculated receptor occupancies comes from the observation that the maximal response to oxotremorine, which must occupy 10-15% of the receptors for maximal response (82), was slightly depressed. Furthermore, the maximal response to the partial agonist, BM 5, which must occupy virtually all the receptors for its maximum response (85), was completely abolished (Table 2). The lower receptor occupancies after exposure to 2 §M of BM 123 and to 5 μM of BM 130 and after the protection experiments were confirmed by the observations that the maximal response to oxotremorine was unaffected and that the response to BM 5 was depressed, the depression roughly paralleling the extent of receptor alkylation. Finally, the calculated receptor occupancies following exposure of the ileum to BM 123 and BM 130 showed general agreement with those estimated by reduction of [^{3}H](-)QNB binding to homogenates. The latter observation contrasts with some recent results obtained in the guinea pig ileum with the irreversible antagonist benzilylcholine mustard. Major discrepancies were found between muscarinic receptor occupancy by benzilylcholine mustard determined from shifts in the dose-response curve to an agonist and from inhibition of [^{3}H]QNB binding (86).

We conclude on the basis of the above results that BM 123 and BM 130 cause a dose-dependent, persistent decrease of sensitivity of the guinea pig ileum to muscarinic agonists, presumably through covalent interaction with the muscarinic receptor. This functional blockade appears to be specific since it is prevented by low concentrations of methylatropine and since it occurs at concentrations that have no nicotinic effects on frog rectus. The mechanism of this apparent blockade has yet to be established. For example, it is not known whether covalent binding of BM 123 and BM 130 results in an active or inactive receptor complex. As previously pointed out (87), it is possible that the receptor remains in an activated state after alkylation by the aziridinium ions. A persistent desensitization could then result in a functional blockade similar to that caused by an irreversibly bound antagonist. In contrast, the irreversible binding of bromoacetylcholine (88) and chloroxymorphamine (89) to nicotinic and opioid receptors, respectively, leads to a sustained tissue response. These agents, however, are believed to bind covalently at a noncritical site near the receptor (88,89). Presumably, BM 123 and BM 130 alkylate a nucleophilic group at the agonist recognition site proper.

5.1.2 Receptor binding properties

In prior studies of the effects of BM 123 on muscarinic receptor binding properties, we noted a reduction in the binding capacities of [^{3}H](-)NMS and [^{3}H](-)QNB in homogenates of the rat cerebral cortex and guinea pig ileum which had been previously incubated with 10 M BM 123 for 20 min at 37° and then washed

extensively. These effects on binding capacity were not accompanied by a significant change in the apparent affinity of [^{3}H](-)NMS or [^{3}H](-)QNB. In the present study, we measured [^{3}H](-)NMS binding in cortical homogenates which had been previously incubated at 37° with a rather high concentration (1.0 mM) of BM 123 for 20 min and 1 hr, and again observed a reduction in binding capacity without an effect on affinity. These results illustrate that BM 123, over a wide range of concentrations, reduces the binding capacity of muscarinic receptors without influencing their affinity, presumably by covalently binding to the recognition site of the muscarinic receptor.

Since low temperature inhibits the formation of the aziridinium ion as well as the covalent binding of the aziridinium ion to the muscarinic receptor, it was possible to determine the affinity of BM 123 and its transformation products for muscarinic receptors by measuring their ability to inhibit [^{3}H]oxo-M and [^{3}H](-)NMS binding competitively. In competition experiments with [^{3}H]oxo-M, the aziridinium ion was 180 and 710-fold more potent than BM 123 and its alcoholic hydrolysis product, respectively. In competition experiments with [^{3}H](-)NMS, the aziridinium ion was 71-fold more potent than both BM 123 and its alcoholic hydrolysis product. These observations are consistent with current pharmacological experiments in our laboratory, which suggest that contractile activity of BM 123 on the isolated guinea pig ileum could be attributed almost entirely, though not completely, to the aziridinium ion.

Like other efficacious agonists, BM 123A appears to discriminate among different agonist subclasses of the muscarinic receptor. Birdsall and coworkers (57,90) have shown that the complex binding properties of muscarinic agonists in rat cerebral cortex can be explained by the existence of 3 classes of binding sites (superhigh, high and low affinity), each having a different affinity for most agonists and equal affinity for most antagonists. Exceptions to this behavior include the selective ganglionic muscarinic agonist McNA343, the selective muscarinic antagonist pirenzepine (91), and some trichloro-derivatives of cis dioxolane having agonistic activity (92). In the present study, we have used low concentrations of [^{3}H]oxo-M and [^{3}H](+)CD to label the superhigh affinity site selectively so that the binding properties of this site could be studied. Previous experiments with [^{3}H]oxo-M (57) and [^{3}H](+)CD (93) have shown that it is possible to label the superhigh affinity site selectively with these [^{3}H]ligands. We estimate that the contribution of the superhigh affinity site to [^{3}H]antagonist binding represents less than 3% of the total binding in washed homogenates of rat cerebral cortex. Consequently, we have assumed that the superhigh affinity sites represent a negligible fraction of [^{3}H]antagonist binding and that measurements of [^{3}H](-)QNB and [^{3}H](-)NMS binding can be interpreted satisfactorily assuming that the high and low affinity sites account for all of the binding in rat cortex.

It has been shown for a number of agonist analogues that efficacy is correlated with the ratio of dissociation constants for the high and low affinity sites (K_L/K_H) (7, 9). In this respect, the binding properties of BM 123A resemble those of a highly efficacious muscarinic agonist since its ratio of dissociation constants for the high and low affinity sites was relatively large when measured by competitive inhibition of [^{3}H]antagonist binding at 0° (K_L/K_H = 95) and by analysis of the kinetics of receptor alkylation at 37° (K_L/K_H = 286).

When measured by competitive inhibition of [^{3}H]oxo-M and [^{3}H](-)NMS binding at 0°, the reversible binding characteristics of BM 123A resembled those of oxo-M. However, there were some significant differences among the binding parameters, the most notable being the value of K_I, which was about 2.5 fold less for the aziridinium ion as compared to oxo-M. In our laboratory pharmacological experiments by Ringdahl have noted that BM 123A was approximately 3 - 4 fold more potent than oxo-M at eliciting contractions of the guinea pig ileum. Thus, the rather minor structural difference between the trimethylammonium group of oxo-M and the aziridinium ring derived from the 2-chloroethylmethylamine group of BM 123, appears to cause small differences in both the pharmacological activity of these compounds as well as their reversible binding characteristics.

The simple one-site model used to describe the competitive inhibition of the alkylation of muscarinic receptors by BM 123 is a simplified approximation since the aziridinium ion as well as oxotremorine and oxo-M discriminate among different agonist subtypes of the muscarinic receptor. Nevertheless, this approximation appears to be self-justified since there is good agreement between the kinetically determined K_I values of the reversible inhibitors and the concentration of the reversible drugs required to occupy 50% of the muscarinic receptors in rat cerebral cortex. The concentrations of atropine, oxotremorine and oxo-M which caused a doubling in the half-time for alkylation of [^{3}H](-)QNB binding sites by BM 123 may be considered as an apparent K_I value (K'_I). This apparent K_I value is larger than the true K_I value by a factor of $1 + A/K_A$ (see equation 18). If we consider the theoretical X_{50} value (2.2 μM) of the aziridinium ion at 37° as an approximation of K_A, then the kinetically determined K'_I values of atropine, oxotremorine and oxo-M (2.5 nM, 1.0 μM, and 4.0 μM) can be corrected to their respective true K_I values of 1.1 nM, 0.44 μM and 1.74 μM. These corrected K_I values are in good agreement with the X_{50} values of atropine and oxo-M measured by competitive inhibition of [^{3}H](-)NMS binding in the present study (0.8 nM and 3 μM, respectively) and with the corrected IC_{50} value of oxotremorine measured by competitive inhibition of [^{3}H]propylbenzilylcholine binding to rat cerebral cortex in previous studies (94). Moreover, atropine, oxotremorine and oxo-M inhibited the alkylation of muscarinic receptors by BM 123 in a manner that was consistent with competitive antagonism. We conclude that the aziridinium ion alkylates the site on the muscarinic receptor where

acetylcholine, atropine and other directly acting muscarinic agents bind.

Our study of the kinetics of muscarinic receptor alkylation over a range of concentrations of BM 123 enabled us to estimate both the affinity of the aziridinium ion and its rate constant for alkylation of the subclasses of agonist binding sites. In our analysis of the kinetics, we have assumed a quasi-equilibrium where the aziridinium ion rapidly equilibrates with the receptor, forming a reversible complex which converts to a covalent complex at a relatively slower rate. This model predicts that the rate of loss of unalkylated receptors should be mono-exponential so long as the change in the concentration of the aziridinium ion during the incubation is not great. The observed rate constant for alkylation of receptors should increase as a Michaelis-Menten-like function of the aziridinium ion concentration. The kinetics of the alkylation of the superhigh affinity site by BM 123 obeyed the simple one-site model described above as demonstrated by the good agreement between the estimates of the affinity of the aziridinium ion measured kinetically at 37° and that estimated directly by competitive inhibition of [^{3}H]oxo-M and [^{3}H](+)CD binding at 0°. The agreement between the two independent estimates made at different temperatures suggests that changing the temperature from 0° to 37° had little influence on the affinity of the aziridinium ion for the superhigh affinity site. This conclusion would appear to be reasonable since we found that changing the incubation temperature from 0° to 37° had no significant influence on the potency with which nonlabelled oxo-M inhibited [^{3}H]oxo-M binding to cerebral cortex when assayed at a [^{3}H]ligand concentration of 1.0 nM (data not shown).

Although the concentration of the aziridinium ion fell to approximately half of its starting concentration after an hour of incubation at 37°, this change in concentration did not produce results much different from those to be expected had the concentration of the aziridinium ion remained constant. In other words, there was general agreement between the estimates of the kinetic parameters of the aziridinium ion for alkylation of the superhigh affinity site when the parameters were determined by fitting all of the data simultaneously to equation 5 and by fitting the kinetic curves individually to an exponential equation and plotting the observed rate constant as a function of the BM 123 concentration. Nevertheless, since we could accurately calculate the decline in the concentration of the aziridinium ion during an incubation at 37°, we felt that fitting the data to equations 5 and 6 would produce the best estimates of the kinetic parameters of BM 123.

The kinetics of the alkylation of [^{3}H](-)QNB binding sites by BM 123 were complex, and could not be described by a simple one-site model. Once occupied by the aziridinium ion, 70 - 75% of the [^{3}H](-)QNB binding sites were alkylated with a half-time of 2.7 min whereas the remaining sites were alkylated more slowly with a half-time of 32 min. Apparently, it is primarily the low

affinity agonist binding site which is alkylated at a faster rate since prior incubation of cortical membranes with a saturating concentration of BM 123 (1.0 mM) for a relatively short time (10.3 min) caused a selective loss of low affinity agonist binding sites as measured by the shift in the oxo-M/[^{3}H](-)NMS competition curve. When the kinetic data showing the loss of [^{3}H](-)QNB binding sites in the presence of various concentrations of BM 123 were analyzed by nonlinear regression analysis, estimates of the dissociation constants of the aziridinium ion for the high and low affinity sites were obtained which were roughly similar to those calculated directly by measuring the competitive inhibition of [^{3}H](-)NMS binding by the aziridinium ion at 0°. The largest discrepancy was in the value of K_L, which was four times larger when estimated kinetically at 37° than that estimated from the results of the BM 123A/[^{3}H](-)NMS competition experiment at 0°. This difference in affinity can probably be attributed to temperature since we have found that increasing the temperature from 0° to 37° caused 2 and 4 fold increases, respectively, in the K_H and K_L values of oxo-M, when estimated by nonlinear regression analysis of oxo-M/[^{3}H](-)NMS competition experiments in rat cerebral cortex.

There are some discrepancies between our data for the alkylation of [^{3}H](-)QNB binding sites by BM 123 and the consequences of the model for two independent sites (equation 6). Firstly, the proportion (75%) of [^{3}H](-)QNB binding sites which were alkylated rapidly by BM 123 at a concentration of 1.0 mM was slightly, though significantly, greater than the estimate of the proportion of low affinity sites (70%) determined by nonlinear regression analyses of the kinetic data obtained at lower concentrations of BM 123 (0.01 - 100 μM). Secondly, the proportion of residual high affinity sites in cortical homogenate which had been pretreated with 1.0 mM BM 123 for 10.3 min was not as great as that predicted from the kinetic analysis. In control tissue, the ratio of high to low affinity sites was 30:70, and following BM 123 treatment (1.0 mM; 10.3 min), the ratio changed to 70:30. However, using equation 6 and the estimates of the kinetic parameters of BM 123, it can be calculated that the ratio of high to low affinity sites should have been 83:17 following exposure to BM 123 (1.0 mM) for 10.3 min. One possible explanation for this discrepancy is that some of the high and low affinity sites in the rat cerebral cortex are interconvertible. Alternatively, it may be that, at high concentrations, BM 123 alkylates nonspecific sites on the muscarinic receptor, causing the affinity of agonists for the primary site to be reduced. However, when we incubated cortical homogenates with BM 123 (1.0 mM) for 1 hr in the presence of atropine (0.1 mM) to protect the primary recognition sites, we were unable to detect any change in the ability of oxo-M to displace [^{3}H](-)NMS binding to the homogenates competitively after extensive washing. It is possible that the high concentration (0.1 mM) of atropine used in the experiment protected both the primary site and the putative nonspecific site as well. It is unlikely that BM 123 alkylates the allosteric site on the muscarinic receptor to which gallamine binds since we found that incubating cortical homogenates with

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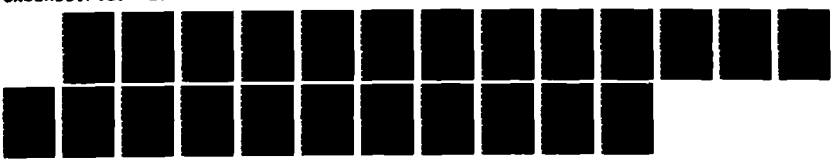

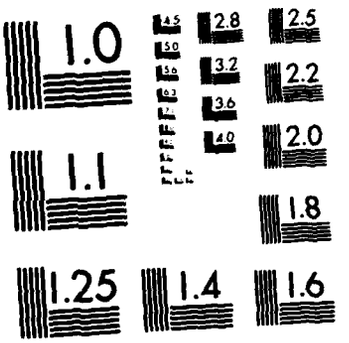

BM 123 (1.0 mM) for 1 hr had no significant influence on the ability of gallamine to inhibit [^{3}H](-)NMS binding to the residual receptors (data not shown). Moreover, after incubating cortical homogenates with BM 123 (1.0 mM) for 1 hr, the affinity of [^{3}H](-)NMS for the residual receptors was unaltered, which might not be expected if BM 123 alkylates the gallamine site.

The relationship between the affinity of the aziridinium ion for the various agonist subtypes of the muscarinic receptor (superhigh, high and low affinity) and the rate at which the aziridinium ion alkylates these sites is reciprocal in nature. In other words, the aziridinium ion alkylated the highest affinity site at the slowest rate whereas the converse was true for the lowest affinity site. The probability that such an ordered, non-random relationship would exist for a group of three distinct and independent binding sites is small. An explanation for this anomaly can be found if we assume that the aziridinium ion, like other muscarinic agonists, induces a conformational change in the receptor when it binds. Accordingly, the aziridinium ion (A) first binds upon the receptor in the ground state (R) and subsequently induces the receptor to assume its activated conformation (R^*); thereby, introducing the possibility that the aziridinium ion alkylates the two conformations at different rates as described by the microscopic rate constants (k', k'') shown below:

$$\begin{array}{ccccc} & & K_1 & & K_\alpha \\ A + R & & AR & & AR^* \\ & & k' & & k'' \\ & & A\text{-}R & & A\text{-}R^* \end{array} \qquad (1)$$

In this scheme, K_1 is the equilibrium dissociation constant describing the binding of the aziridinium ion to the ground state of the receptor and K_α is the unimolecular equilibrium constant describing the isomerization ($K_\alpha = XR/XR^*$). The overall experimental dissociation constant K_A is given by:

$$K_A = \frac{K_\alpha K_1}{K_\alpha + 1} \qquad (2)$$

During an incubation of the aziridinium ion with receptors, the loss of unalkylated receptors in time is given by:

$$Y = e^{-kpt} \qquad (3)$$

where Y is the proportion of unalkylated receptors, t is time, and p is receptor occupancy by the aziridinium ion ($p = A/(A + K_A)$). The overall experimental rate constant k for alkylation is given by:

$$k = \frac{k'K_\alpha + k''}{K_\alpha + 1} \qquad (4)$$

Birdsall et al. (57) have presented evidence that the differences among the dissociation constants of most agonists for the

superhigh, high and low affinity sites are due to differences in the propensity with which agonists isomerize these receptors and not to major differences in the conformations of the ground states of these receptors. According to this postulate, the dissociation constants (K_{SH}, K_H and K_L) of agonists are given by:

$$K_{SH} = \frac{K_{\alpha SH}K_1}{K_{\alpha SH} + 1} \quad (5)$$

$$K_H = \frac{K_{\alpha H}K_1}{K_{\alpha H} + 1} \quad (6)$$

$$K_L = \frac{K_{\alpha L}K_1}{K_{\alpha L} + 1} \quad (7)$$

where $K_{\alpha SH} < K_{\alpha H} < K_{\alpha L}$. If we assume that the aziridinium ion alkylates the activated conformation at a slower rate than that at which it alkylates the ground conformation ($k'' < k'$), then it follows from equation (24) that the magnitudes of the experimental rate constants for alkylation of the superhigh, high and low affinity sites must have the following rank order: $k_{SH} < k_H < k_L$.

There is no way to calculate explicit values of K_1, $K_{\alpha SH}$, $K_{\alpha H}$, $K_{\alpha L}$, k' and k'' from the data; however, we can make some limiting assumptions. Since the ratios of dissociation constants (K_L/K_H, K_H/K_{SH}) of the aziridinium ion for subtypes of the muscarinic receptor exceed the respective ratios of rate constants (k_L/k_H, k_H/k_{SH}), then it follows that the aziridinium ion alkylates both ground and activated conformations of the receptor at finite rates ($0 < k'' < k'$) and that $K_{\alpha L}/K_{\alpha SH} > k'/k''$. If we speculate that the overall experimental dissociation constant of the aziridinium ion for the low affinity agonist site is similar to K_1 ($K_{\alpha L} \geq 1$), then it follows that $k'' \geq k_{SH}$ and $k' \geq k_L$.

The model described above provides a simple explanation for the quantitative relationship between the rate constants and dissociation constants of the aziridinium ion. It has its origins in previous ideas about the significance of agonist receptor heterogeneity in the rat cerebral cortex, and states that the tighter binding of the aziridinium ion to the activated conformation of the muscarinic receptor hinders the covalent binding of the aziridinium ion to a nucleophile on the receptor. It is hoped that the selectivity of the aziridinium ion for different agonist binding sites may provide a useful tool for investigating agonist receptor heterogeneity.

5.1.3 Effects of DFP on presynatpic acetylcholine release

The results of the present experiments show that a significant effect of chronic depression in AChE activity by the anticholinesterase, DFP, is disinhibition of ACh evoked release from presynaptic neurons. This is consistent with a model in which muscarinic autoreceptors inhibit ACh release. As was previously established, elevated levels of ACh during chronic DFP treatment (17) lead to downregulation of mAChRs (22,24,93). In the present situation a consequence of elevated ACh may be downregulation of muscarinic autoreceptors resulting in disinhibition of ACh release from presynatpic neurons. We have already shown that chronic treatment with DFP does not alter the synthesis of ACh in brain synaptosomes (21), thus placing emphasis in the present experiment on the release of ACh. This would result in the significantly elevated rates of evoked release from the myenteric plexus of the chronically treated animals in the present experiment when compared with release from the plexus of control subjects.

The results are inconsistent with the possibility we suggested earlier that chronic treatment with anticholinesterase could result in adaptive changes in receptor concentrations at presynaptic as well as postsynaptic muscarinic sites (17). However, they are consistent with the fact that elevated levels of ACh develop and are maintained during such chronic exposures.

The results are also consistent with time parameters reported by Ehlert et al. (93) for maximum decreases in [^{3}H]QNB binding in brain during a DFP regimen similar to that used in the present experiment. They found no significant differences in densities of mAChRs after 4, 10, 14 and 30 days of chronic treatment. Our present results show no significant differences in stimulated ACh release among treatments beyon 4days, i.e. at 6 and 10 days.

When viewed within the general context of results from other experiments on effects of acute and chronic DFP treatments, the rpesent results suggest a sequence of changes in cholinergic function. Significant increases in brain ACh occur within 4 hr of decreases in AChE activity. Elevated ACh levels could initiate processes involved in downregulation of mAChRs which are significant in 1 day and reach their peak in about 4 days, being maintained at peak levels during continued decrease in AChE activity. Downregulation would reduce activity of postsynaptic neurons, while maintaining suprnormal rates of ACh release from presynpatic sites. The latter would be counterproductive to the maintenance of normal cholinergic relationships within the synapse. Despite this, the fact is that ACh levels in brain do indeed remain steadily at 15% above control values during chronic DFP regimen (17).

The present experiment was motivated in part by our long term interest in how homeostatic controls at molecular levels may be associated with adaptive changes in the behavior of the total,

intergrated organism. On the basis of pharmacological evidence we earlier proposed the concept of a finely-tuned process involving downregulation of postsynaptic mAChRs (20), a concept which gained more direct support when receptor binding techniques were applied. Later we suggested the possibility that homeostatic processes involving muscarinic autoreceptors might also be involved in adaptive behaviors (17). Raiteri et al (95) have presented evidence for regulatory changes in synaptosomal autoreceptors in response to chronic treatment with paraoxon. Similar suggestions have been made about behavioral effects modulated by other receptor mechanisms, e.g. catecholaminergic in which behavioral facilitation was hypothesized as "involving probably the progressive disappearance of an inhibiting mechanism mediated by autoreceptors (96, p681)." The present results support an alternative role for presynaptic mAChRs, i.e. that, following decreases in AChE activity, their downregulation leads to greater release of ACh which further modulates the downregulation of postsynaptic mAChRs. The downregulation tames some 4 days to stabilize and continues as long as the primary stimulus, decreased AChE activity, persists. Such a series of events could occur concomitantly with changes in behavior. The recovery of different behaviors to pretreatment states, i.e. development of tolerance, could be related to their requirements for different level of downregulation.

5.2 BEHAVIORAL AND PHYSIOLOGICAL STUDIES

5.2.1 EFFECTS OF BM 130

Neurochemical and neuropharmacological evidence from our earlier studies of effects of mustard analogs of oxotremorine on animal models in vitro and in vivo have indicated that these compounds act initially as agonists and thereafter may produce a sustained resistance to muscarinic agonists. The prolonged action appears to result from an irreversible interaction between mAChRs and the active species of these compounds, i.e. their aziridinium ions. In view of the extensive evidence that the cholinergic neurotransmitter system is involved in a wide variety of behavioral and physiological functions of intact organisms (18), it is a reasonable hypothesis that effects of the oxotremorine mustards should be reflected in these variables. The present series of experiments was designed to test this hypothesis.

(1) Cholinomimetic effects of acute administration

Results of the present experiments clearly demonstrated that, when acute effects of BM 130 occurred, they were consistent initially with well-established effects produced by known cholinergic agonists. The general signs of such agents appeared centrally and peripherally following administration of BM130, but after injections of BM 130A, its quaternary aziridinium ion, only the peripheral effects were seen. Full recovery from these signs occurred remarkably rapidly, within 5-15 min under the majority of conditions and doses studied. Another well-documented effect of cholinergic agonists, i.e. production of hypothermia, was also

clearly observable and dose-dependent. The maximum hypothermic effect occurred soon after injection of BM 130 and disappeared well within 60 min.

Acute effects of BM 130 were not seen in any of the other major behavioral variables. All had been measured 30 min after drug treatment in order to avoid possible interference by tremor, salivation, chromodacryorrhea or other general signs described above. The only significant effects at this time point were decreases in nociceptive thresholds induced by BM 130A, but not by BM 130. Because nociception is heavily dependent upon sensory-perceptual processes (80), the hyperalgesia may have resulted from differential effects of BM 130 and BM 130A on peripheral sensory processes related to the restriction of the quaternary form to peripheral sites of action. There were no acute effects of either BM 130 or BM 130A on CAR, which was dependent upon aversive stimulation (electric shock) for its reinforcement. Likewise, there was no evidence that the two compounds had acute effects on the operant behavior, FR_5, which was maintained by appetitive reinforcement (water intake).

These results are consistent with one of our predictions, i.e. that the initial effects of BM 130 should be cholinomimetic. They also show that this direct effect is of limited duration, being evidenced as briefly as 5-15 min for some of the behavioral and physiological variables measured and never longer than 30-60 min in all others. This fact raises questions about the nature of the compensatory processes by which the behavioral and physiological variables recover from the initial effects despite the irreversible interaction of the compound with the mAChRs ion. These must be processes which have very short lead times in being activated and are capable of compensating for a considerable range of mAChRs occupied. It has been suggested that the simplest model in such situations would be one in which manipulation of the neurotransmitter system, e.g. BM 130 occupying mAChRs, induced not only the primary behavioral and physiological effects, but also initiated a compensatory process (1). Our present research design included studies of effects of repeated BM 130 injections at relatively short intervals, i.e. 15 min, as a means of probing such a homeostatic process, should it occur.

(2) Effects of repeated administration

The outcome of repeated administration was a decrease in magnitude and eventual disappearance of effects. It has long been known that sustained exposure to ACh or other muscarinic agents may induce desensitization. Resistance to muscarinic agents can also occur because of a decrease in the number of receptors, for example following downregulation induced by anticholinesterase treatment (22). It is not clear which of these two mechanisms might be responsible for the resistance to the agonist actions of oxotremorine or BM 130 which developed when the latter was administered repeatedly. If the muscarinic

receptor remains in an active state after alkylation by BM 130, desensitization would be expected to develop; it it is left in an inactive state, resistance to muscarinic agonists would develop because of a decreased number of functioning receptors. Further experiments will be required to discriminate between these alternatives, and it is possible that both mechanisms may operate, the net effect depending on the kinetics of desensitization and of receptor replacement. These are likely to differ at different sites of action, and may thus explain differential effects of BM 130 among several muscarinically mediated responses.

(3) Prolonged effects of acute administration

The second major prediction from our general hypothesis was that initial cholinomimetic effects of BM 130 should be followed by sustained resistance to cholinergic agonists, i.e. that BM 130 would "protect" against the actions of direct and indirect agonists. This prediction was tested by subjecting animals to oxotremorine challenges at weekly intervals following single injections of BM 130, BM 130A or saline. In none of the measures taken did animals injected with BM 130A or saline give evidence of resistance to the muscarinic challenges. Those administered BM 130 did show sustained resistance in some of the variables measured. The resistance was statistically significant at high doses and appeared to be dose dependent over the range studied.

Results from experiments with the FR_5 operant response indicated that whether or not resistance was observed was at least in part a function of the dose of oxotremorine used during the challenge series. There were no differences between the three treatment groups with doses of 1 μmol/kg, but the group injected with the highest dose of BM 130 was significantly more resistant than the others when the challenge dose was 0.25 μmol/kg. This suggests that different behavioral and physiological variables differ in response thresholds. A consequence would be that demonstration of sustained resistance following administration of compounds such as BM 130 would be critically dependent upon the dose of cholinergic agonist involved in challenging the system.

This possibility may also affect estimates of the duration of the sustained resistance. In our present experiments the evidence pointed to resistance persisting for 2-3 weeks in measures such as general signs and nociception when the oxotremorine challenge was 1 μmol/kg. In other measures, e.g. FR_5 responding, resistance to oxotremorine was found to continue after 4 weeks when 0.25 μmol/kg was used as the challenge dose, although no differential resistance had been seen following a higher dose administered earlier.

Determining duration of prolonged effects of acute administration may also be confounded by the development of tolerance to oxotremorine during the series of challenges. If this had occurred in the present experiments, it should have been

evidenced in measures of the saline control groups. Comparisons of data for saline groups provided no evidence that such tolerance had occurred in the general signs or in any measures of nociceptive thresholds and FR_5 responding. However, performance of the CAR by the Saline group did show a systematic decrease in effects of oxotremorine during the challenge series. Why tolerance might develop in this behavior and not in others is unclear. Nevertheless, the discrepancy indicates the importance of considering the possibility of such a confounding effect in experiments involving multiple challenges to neurotransmitter systems.

Evidence from our results supports the conclusion that, within the range of doses studied, the sustained effects of BM 130 cannot be attributed to neuromuscular impairment. A measure frequently used to observe changes in motor aspects of behavior is time taken to make a response, impairment appearing as increases in response time. No such effects were seen in times taken to make the learned response, Ra, in the CAR situation after acute treatments with various doses of BM 130 and at the supraliminal shock intensities used. There also were no differences between groups in making the quite different motor responses of lever-pressing under the FR_5 conditions. No impairment or reflexes was noted during the observations of general signs. The general incapacitation, "time out," which follows treatment with cholinergic agonists did not appear.

5.2.2 Effect of BM 123

It is premature to draw final conclusions about effects of BM 123 on behavioral and physiological function, until further analysis of data is completed. However, the results now available stongly suggest that the time courses of changes induced by BM 123 vary widely from physiological functions affected alomost immediately and returnin to normal within minutes to cognitive processes requiring days to recover. There also appears to be evidence for the development of tolerance in some, but not all functions. Comparisons of effects elicited by oxotremorine and by BM 123 suggest that the latter produces a sustained change in receptor-mediated events which would be expected of an irreversible ligand. This sustained change may "protect" against behavioral and physiological effects of exposure to direct or indirect cholinergic agonists.

5.2.3 Effect of withdrawal from chronic DFP

Further analyses of data already collected and additional experiments are necessary before a full story of withdrawal effects can be written. Analyses so far completed provide evidence consistent with that reported by us and by other investigators relating to the acute and chronic effects of the administration of DFP. Significant initial changes are followed by the development of tolerance as the duration of treatments continued. This pattern is differnetially characteristic of

behavioral and physiological functions in which the cholinergic system is involved.

Having established that the DFP regimen produced characteristic effects (17,69,97), our attention focused on changes in behavioral and physiological functions after withdrawal of the treatment, a condition about which very little information is yet available. Analyses of preliminary results give some hints about the directions that final conclusions may take. Recovery to normal levels of AChE activity in smooth muscle appears to occur more rapidly than recovery in brain. Evoked release of ACh tends to decrease systematically toward control levels as time after DFP withdrawal increases. In general physiological functions develop tolerance during the treatment regimen, yet show no further changes when the several biochemical changes mentioned above are occurring, i.e. the two classes of biological events appear not to be highly correlated. Some unexpected effects are also being observed. For example, two major behavioral variables, nociception (algesia) and general activity, appear to undergo extended perios of supersensitivity after the beginning of the withdrawal period. Because of the importance to understanding how the sequelae of exposures to antiAChEs may affect the capabilites of living organisms to cope with their ever changing environments, the two series of experiments on withdrawal phenomena are being extended.

6. RECOMMENDATIONS

Although the first year of the contract has seen completion of several extensive series of experiments, it is still too early in the overall program to make recommendations other than that the program continue as originally planned. However, research results already available support the specific recommendation that particular attention should be given to studying possible applications of the sustained resistance of behavioral and physiological functions to cholinergic agonists during the prolonged effects of the irreversible oxotremorine analogs, BM 123 and BM 130. Such compounds might provide "protection" against such indirect cholinergic agonists as the organophosphorus anticholinesterases. With completion and approval of the required research facilities, experiments on adaptation to chronic administration of the anticholinesterases, DFP and soman, and the effects of their abrupt withdrawal can begin.

7. ILLUSTRATIONS AND TABLES

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