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The Hypoxia Screen as an Indicator of Alterations in the Number of Centeral Cholinergic Recpetors Inuduced by Chronic Acetylcholinestrerase Inhibition

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THE HYPOXIA SCREEN AS AN INDICATOR OF ALTERATIONS IN THE NUMBER OF CENTRAL CHOLINERGIC RECEPTORS INDUCED BY CHRONIC ACETYLCHOLINESTERASE INHIBITION

Kathleen B. Wallace

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ABSTRACT

Chronic administration of diisopropyl fluorophosphate (DFP) at 72 hour intervals for a total of 14 doses resulted in a reduction in the number of central muscarinic receptors in mice as measured by the specific binding of [^{3}H]Quinuclidinyl benzilate (QNB) to cortical tissue homogenates (pmole/mg protein). The number of cholinergic receptors were reduced significantly by 12% in DFP treated animals (0.687 $\pm$ 0.018 pmoles/mg) as compared to vehicle treated controls (0.781 $\pm$ 0.016 pmoles/mg) after the sixth dose of DFP (Day 17). The decrease in the number of central cholinergic receptors was maximal after the 8th dose of DFP (Day 23), and central cholinergic receptor number remained significantly depressed through the 14th dose of DFP. DFP administration was terminated after the 14th dose (Day 40), and the number of central cholinergic receptors increased to 0.853 $\pm$ 0.013 pmole/mg after 6 days and returned to pretreatment levels (0.934 $\pm$ 0.019 pmoles/mg) 13 days after the last dose of DFP. This study demonstrated that the number of central muscarinic receptors could be reduced by chronic DFP treatment, and binding could return to control levels after drug treatment was terminated.

Hypoxia survival times were used to assess central cholinergic activity. Therefore the next study was designed to examine the relationship between DFP-induced alterations in central muscarinic receptor number and hypoxia survival times in mice during and following chronic administration with DFP at 72 hour intervals.

At the first time point examined (Day 5, after the second dose of DFP) hypoxia survival times were significantly increased in DFP treated

mice while receptor numbers were not significantly altered. Survival times of mice in hypoxic conditions were significantly increased after the 6th (Day 17), 12th (Day 35) and 14th doses (Day 41) of DFP, whereas the number of central cholinergic receptors was significantly reduced. The data from these three time points demonstrated that there is an inverse relationship between hypoxia survival times and the number of central muscarinic cholinergic receptors. Hypoxia survival times were not significantly altered 20 days (Day 60) after the last dose of DFP was administered, but the number of muscarinic receptors remained significantly depressed. On Days 5 and 60 the inverse relationship between hypoxia survival times and the number of central muscarinic receptors was not apparent.

These studies demonstrate that the number of central cholinergic receptors can be altered during and following chronic DFP administration, but that hypoxia survival times were not a clear indicator of the number of central muscarinic receptors.

INTRODUCTION

There are few experimental animal models which reflect the physiological deficits characteristic of senile dementia. Development of an animal model is necessary if new drugs to treat senile dementia are to be developed. A strong link between senile dementia of the Alzheimer type in humans and the presence of cholinergic hypoactivity has been established (1). The objectives of this study are to develop an animal model with a chemically induced cholinergic subsensitivity (reduction in the number of central muscarinic receptors) similar to that observed in senile dementia patients and to evaluate the hypoxia screen as an indicator of these chemically induced changes in receptor numbers.

Cholinergic Pharmacology

Acetylcholine is a neurotransmitter in the brain. The synthesis of acetylcholine (2) requires the activation of acetate to form acetyl CoA, which then combines with choline in the presence of the enzyme, choline acetyltransferase to form acetylcholine and CoA. Acetylcholine is stored in membrane limited synaptic vesicles. A nerve impluse upon reaching a nerve ending causes these vesicles to fuse with the synaptic membrane and empty their contents into the synaptic cleft. Acetylcholine then diffuses across the synaptic cleft and binds to specific acetylcholine receptors on the postsynaptic neuron to produce membrane depolarization. Postsynaptic acetylcholine receptors in the brain are classified as muscarinic receptors (Figure 1). Soon after this neurotransmitter receptor interaction, acetylcholine is hydrolyzed to acetic

FIGURE 1

Schematic representation of a cholinergic neuron.

(1) Activation of acetate to form acetyl CoA.

(2) The enzyme choline acetyltransferase catalyzes the conversion of Acetyl CoA and choline to form acetylcholine.

(3) A nerve impulse reaches the nerve ending causes the synaptic vesicle to fuse with the synaptic membrane.

(4) Synaptic vesicle releases acetylcholine into the synaptic cleft.

(5) In the synaptic cleft, acetylcholine is hydrolyzed to acetic acid and choline by acetylcholinesterase.

(6) Acetylcholine that successfully crosses the synaptic cleft binds to the postsynaptic muscarinic receptor.

(7) Postsynaptic muscarinic receptor.

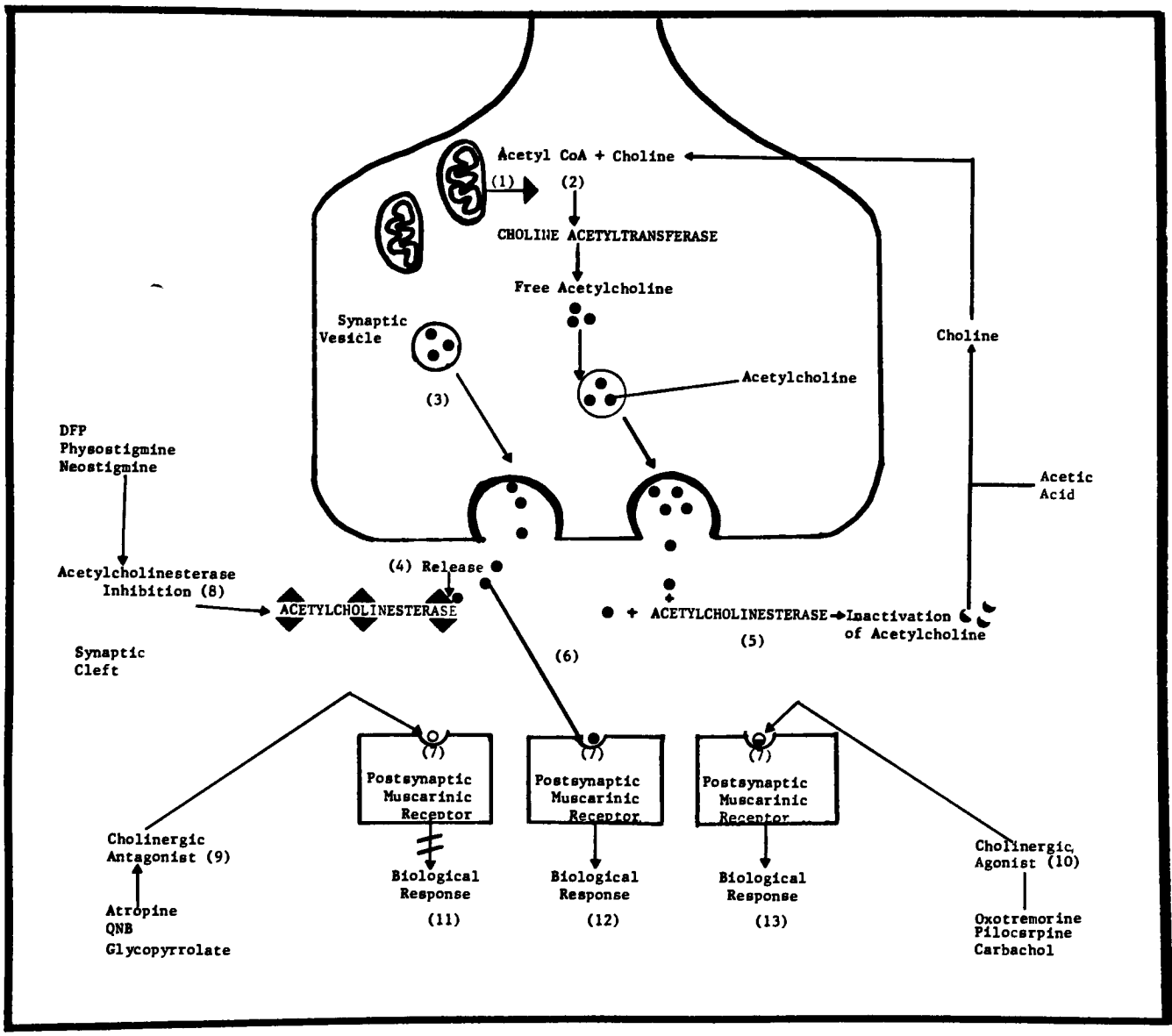
(8) Drugs such as DFP, neostigmine and physostigmine inhibit acetylcholinesterase.

(9) Drugs such as atropine, glycopyrrolate, and QNB are cholinergic antagonists and bind at the postsynaptic muscarinic receptor, but have no intrinsic activity.

(10) Drugs such as oxotremorine, pilocarpine and carbachol are cholinergic agonists which mimic the action of acetylcholine.

(11) Receptor bound to a cholinergic antagonist which results in no biological response.

(12) & (13) Acetylcholine or cholinergic agonist bound to a muscarinic receptor which initiates a biological response.


Acetyl CoA + Choline
(1)
(2)
CHOLINE ACETYLTRANSFERASE
Free Acetylcholine
Synaptic Vesicle
Acetylcholine
(3)
Choline
DFP
Physostigmine
Neostigmine
Acetic Acid
Acetylcholinesterase Inhibition (8)
(4) Release
ACETYLCHOLINESTERASE
+ ACETYLCHOLINESTERASE → Inactivation of Acetylcholine
(5)
Synaptic Cleft
(6)
(7)
Postsynaptic Muscarinic Receptor
Postsynaptic Muscarinic Receptor
Postsynaptic Muscarinic Receptor
Cholinergic Antagonist (9)
Atropine
QNB
Glycopyrrolate
Biological Response
(11)
Biological Response
(12)
Biological Response
(13)
Cholinergic Agonist (10)
Oxotremorine
Pilocarpine
Carbachol

acid and choline by acetylcholinesterase located on the postsynaptic membrane.

Drugs can impair, mimic, or enhance the cholinergic neurotransmission process. The synthesis of acetylcholine can be enhanced by a choline supplement to the diet while the hemacolinium compound, HC-3, interferes with acetylcholine synthesis by blocking the uptake of choline by the presynaptic neuron (2). The release of acetylcholine is altered by Ca^{+2} and Mg^{+2} ions and by the botulinus toxin (2). Drugs which have the ability to inhibit acetylcholinesterase reversibly and irreversibly are called anticholinesterase agents (Figure 2). Physostigmine and neostigmine are reversible acetylcholinesterase inhibitors, while diisopropyl fluorophosphate (DFP), an organophosphate (Figure 3), is an irreversible inhibitor (2,3). Reversible acetylcholinesterase inhibitors acetylate the enzyme and form a transient complex with the surface of the enzyme. The drug then competes with acetylcholine for the enzyme's active site. The acetylated enzyme can react with water to reactivate the enzyme. The irreversible acetylcholinesterase inhibitor phosphorylates the enzyme making the enzyme-inhibitor complex irreversible. In either mechanism of action, acetylcholinesterase inhibitors slow down or prevent destruction of acetylcholine, thus resulting in increased levels of neurotransmitter in the synaptic cleft.

A number of drug interactions can take place at the muscarinic receptor on the postsynaptic membrane (Figure 1). Drugs which bind to this receptor and mimic the action of acetylcholine are called cholinergic agonists or cholinomimetics. Examples of cholinergic agonists are pilocarpine, oxotremorine and muscarine (2). Drugs which block

FIGURE 2

Effect of acetylcholinesterase inhibition on acetylcholine hydrolysis. Steps invovled in the hydrolysis of acetylcholine (ACh) by acetylcholinesterase (AChE) (I), and in the inhibition of AChE by carbamyl ester (II), and organophosphorus (III) agents.*

I. The substrate, ACh, combines with an active unit of the enzyme to form a complex (A) by electrostatic attraction between the N^+ atom of the choline moiety and the anionic site of the enzyme, and the electrophilic C atom of the carboxyl group and a protonated acidic group (-G-H) of the esteratic site; choline is then split off (step 2), leaving the acetylated enzyme (B), which reacts rapidly with water (step 3) to produce acetic acid and the regenerated enzyme.

II. Neostigmine and related ammonium- or amino-carbamyl esters react with the enzyme in the same manner as does the substrate (step 1 and 2); however, the dimethylcarbamoyl enzyme (II-B) reacts with water (step 3) at less than a millionth the rate of the corresponding acetylated form (I-B).

III. DFP and similar organophosphorus inhibitors react only at the esteratic site to form a phosphorylated enzyme; in the case of diisopropylphosphoryl-AChE (III-B), essentially no spontaneous hydrolytic reactivation occurs.

Heavy, light and dash arrows represent extremely rapid, intermediate, and extremely slow or insignificant reaction velocities, respectively.

*See Reference 3.

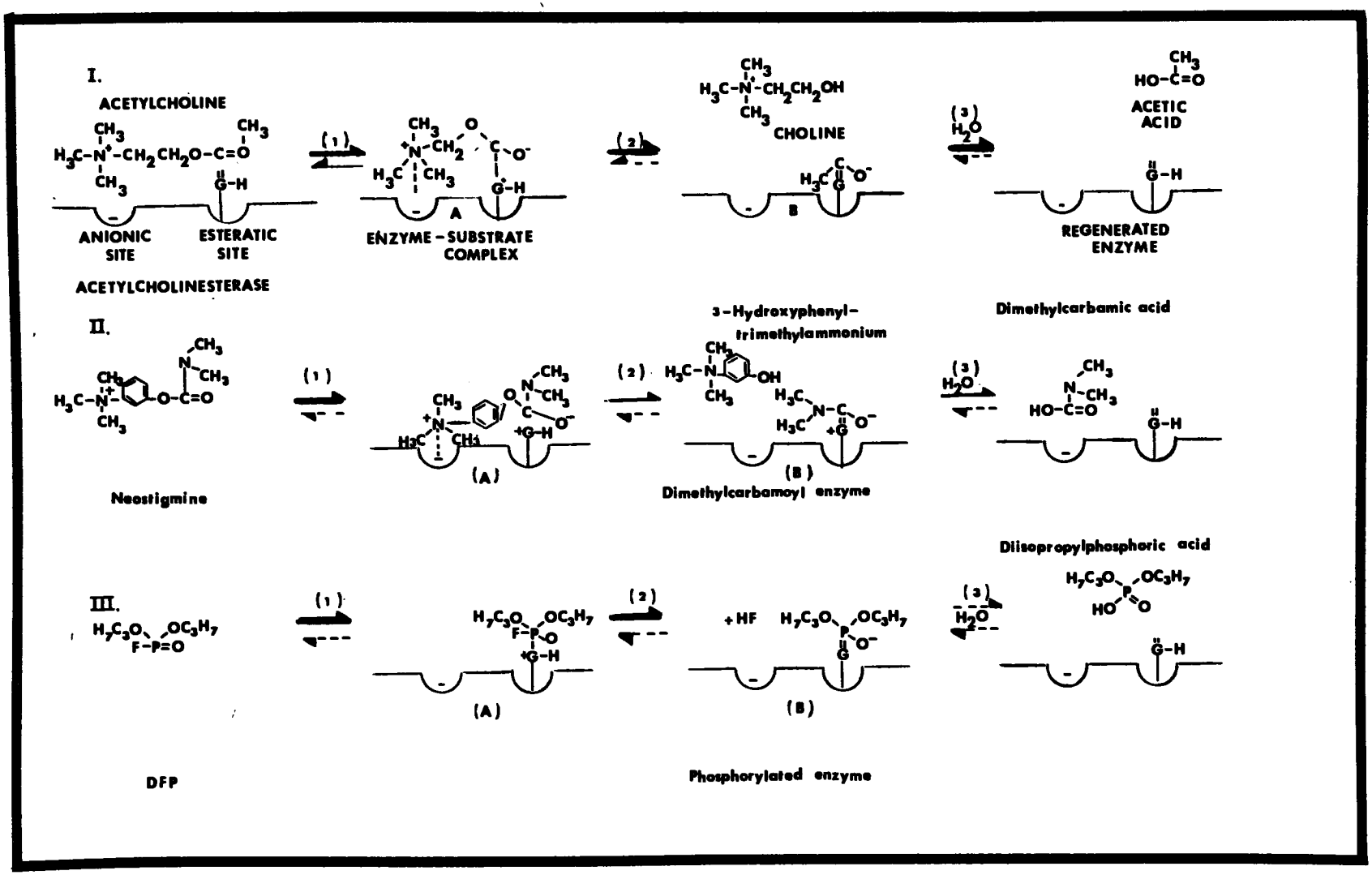
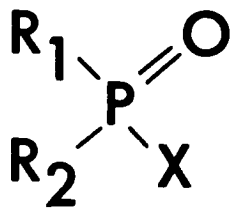

I.
ACETYLCHOLINE
ANIONIC SITE
ESTERATIC SITE
ACETYLCHOLINESTERASE
(1)
ENZYME – SUBSTRATE COMPLEX
A
(2)
CHOLINE
B
(3)
H_2O
ACETIC ACID
REGENERATED ENZYME
II.
Neostigmine
(1)
(A)
(2)
3-Hydroxyphenyl-trimethylammonium
Dimethylcarbamoyl enzyme
(B)
(3)
H_2O
Dimethylcarbamic acid
III.
DFP
(1)
(A)
(2)
+HF
(B)
Phosphorylated enzyme
(3)
H_2O
Diisopropylphosphoric acid

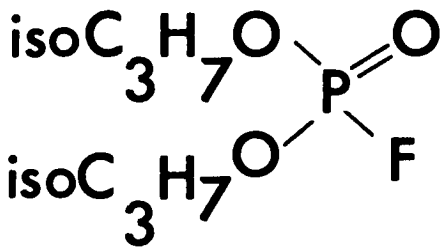
FIGURE 3

Chemical structures of organophosphates and DFP.

Organophosphorus agents



DFP



the receptor by binding at the site but have no intrinsic or biological activity are muscarinic antagonists or cholinolytics. Compounds included in this class of inhibitors are quinuclidinyl benzilate, atropine, glycopyrrolate, and scopolamine (2).

Relationship Between Disease States and Central Cholinergic Activity

The dementias constitute one of the most prevalent groups of diseases today. The clinical state of dementia is characterized by the gradual impairment of memory and learning, loss of intellectual and cognitive functions and disorientation (1). There are approximately one million persons in the United States with dementia. Dementia is one of the most common syndromes in the elderly, afflicting 5% of the over 65 year old population in the United States and Northern Europe (4). As the average age of individuals in the population increases, this phenomenon of dementia could be extrapolated to show a tripling of the incidence of this syndrome by the year 2050.

Historically, the term Alzheimer's disease referred exclusively to presenile dementia (progressive dementia in patients under 65 years of age), whereas the term senile dementia was applied to those 65 and over. Because the two conditions are clinically, neurologically, pathologically, and biochemically alike they are now believed to be the same disease (5). Alzheimer's disease is named for Alois Alzheimer, who in 1906 first described this condition and recognized it as a disease (1). This disease is characterized by neuropathological and neurochemical alterations in areas of the brain associated with memory (6). Neuropathological effects include gross atrophy of the cerebral cortex and degeneration of the neurons in the hippocampus which play an important role in memory function (6).

Recently, the roles of acetylcholine and cholinergic function has been linked to memory and learning tasks (7). Impaired cholinergic function may be an important contributor to geriatric memory deficits. Neurochemical alterations associated with Alzheimer's disease involve a general cholinergic hypoactivity characterized by decreased activity of cholineacetyltransferase, acetylcholinesterase, and decreased muscarinic receptor binding (7-9). This age related decrease in central cholinergic activity is particularly severe in patients with senile dementia, but may be an underlying cause of other memory disorders in the aged.

Further evidence for the relationship between memory and central cholinergic activity is supported by numerous pharmacological studies in man showing a decrement in memory processes following administration of cholinergic antagonists and improvements in some memory functions following administration of cholinergic agonists (10-12).

Animal Models For Senility

No known animal model mimics all or even most of the behavioral, neurochemical and neuropathological defects of senile dementia (13). Investigators have studied certain of these defects in old animals of a variety of species in an attempt to identify characteristics that are in one way or another similar to the changes characteristically seen in the senile human. For example, depressed acetylcholine synthesis has been detected in senescent C57BL/6J and BALB/c mice (14). This decrease has been correlated with behavioral deficits and may contribute to brain dysfunction accompanying senescence (14). However, even this approach has been hampered by the limited supply of naturally

aged animals and the expense of maintaining such colonies for the extended periods of time required for study.

Cholinergic subsensitivity is one characteristic of senile dementia in humans that is also in evidence in some species of naturally aged animals (13). Cholinergic subsensitivity can be defined as a condition in which there is a reduction in the number of central muscarinic cholinergic receptors (Figure 4). Patton and Rang (15) were the first to identify the central muscarinic acetylcholine receptor by demonstrating the presence of a high affinity binding sites for reversible tritiated muscarinic antagonists. [^{3}H]Quinuclidinyl benzilate (QNB), a muscarinic antagonist, was described by Yamamura and Snyder to have high affinity and specificity for muscarinic binding sites in rat brain homogenate (16). [^{3}H]QNB binding has been utilized in a number of studies to assess central cholinergic activity in experimental animals (17-20).

A drastic reduction in the time required to develop cholinergic subsensitivity is observed when experimental animals are chronically treated with DFP (17-18) compared to waiting for development of cholinergic subsensitivity in naturally aged animals. Numerous studies (17-19) have confirmed that chronic treatment of experimental animals with DFP inhibits cholinesterase activity, increases acetylcholine levels, and decreases the number of central muscarinic receptors. Ehlert et al., (20) suggested that DFP-induced alterations in muscarinic binding in the brain are caused by acetylcholinesterase inhibition and the resulting accumulation of acetylcholine at the cholinergic synapse. Those animals receiving chronic DFP treatment showed a

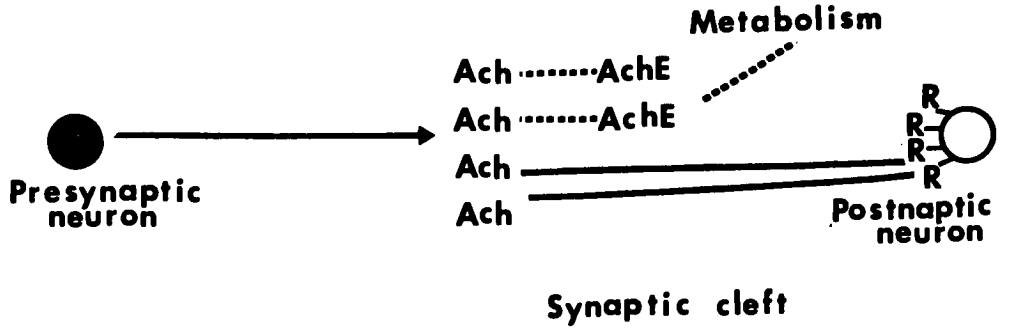
FIGURE 4

Schematic representation of DFP-induced cholinergic subsensitivity.

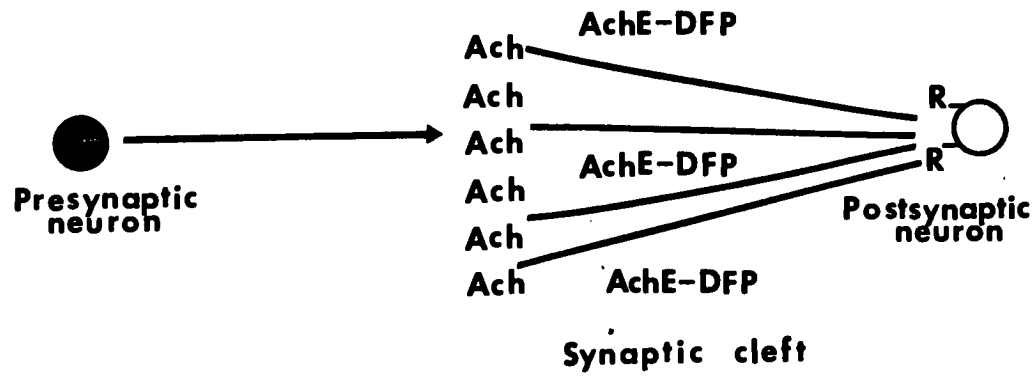
1. Cholinergic transmission-normal cholinergic transmission with acetylcholine (ACh) inactivated by acetylcholinesterase (AchE).

2. DFP Subsensitivity-DFP inactivated acetylcholinesterase (AchE), acetylcholine (Ach) accumulated in synapse, resulting in a reduction in the number of muscarinic postsynaptic receptors.

1. Cholinergic Transmission



2. DFP Subsensitivity



25-30% reduction in the number of central muscarinic acetylcholine receptors. Thus, it appears that chronic treatment with DFP creates a cholinergic hypoactivity similar to the cholinergic subsensitivity in humans. These animals with artificially induced cholinergic subsensitivity may provide a suitable drug induced "senility" model to screen drugs for potential pharmacological activity in the treatment of certain aspects of senile dementia.

Screens Used to Assess Central Cholinergic Activity: Hypoxia Test

1. Relationship Between Survival Time and Central Cholinergic Activity

Centrally acting cholinergic agents have been demonstrated to profoundly alter survival times of animals exposed to hypoxia (atmosphere 96% nitrogen, 4% oxygen). Under this condition death is due apparently to impairment of cerebral function ultimately resulting in respiratory arrest (22).

Several lines of evidence suggest that survival times under hypoxia are mediated by a central cholinergic mechanism. Physostigmine, a reversible acetylcholinesterase inhibitor, causes a dose related increase in survival time of experimental animals during hypoxia (21-23). This effect appears to be due to the accumulation of acetylcholine at central cholinergic synapses (21). The effect of physostigmine on survival time is not related to the drug's action on peripheral cholinergic synapses, since the effect is not mimicked by neostigmine, a reversible acetylcholinesterase antagonist that does not penetrate the blood brain barrier (21). Further studies demonstrate that the peripheral cholinergic antagonist, glycopyrrolate, does not prevent the protective effect of physostigmine in the hypoxia test (21). In contrast to

glycopyrrolate, atropine a cholinergic antagonist which can pass the blood brain barrier, did reduce physostigmine enhanced survival times (21). These studies provide evidence that enhanced survival is a central cholinergic phenomenon since manipulation of the peripheral cholinergic nervous system has no effect on hypoxia survival times (Table 1).

2.- Mechanism By Which Central Cholinergic Activity Alters Survival Times In The Hypoxia Test

Scremin et al. (24) have shown that cholinergic drugs can significantly alter cerebral blood flow and thus may alter survival times by increasing oxygen transport to the central nervous system. By measuring local blood flow in the cerebral cortex of rats they demonstrated increases in cerebral blood flow upon administration of cholinomimetic drugs (24). Atropine was shown to block cerebral vasodilation stimulated by cholinomimetics.

Gibson and Blass (25) hypothesized that a reduction in oxygen supply to the brain during hypoxia reduces the synthesis of acetylcholine in vivo. This reduction in acetylcholine synthesis is related to an impairment of carbohydrate oxidation. Pretreating animals with physostigmine delays death from hypoxia (25), which suggests that hypoxia interferes with the synthesis of acetylcholine in the brain. Thus, although several mechanisms may contribute to alterations in hypoxia survival times, it appears that these mechanisms are linked to the central cholinergic system.

TABLE 1: EFFECT OF CHOLINERGIC DRUGS ON CHOLINERGIC NEUROTRANSMITTER LEVELS AND HYPOXIA SURVIVAL TIMES*

DRUG	SITE OF ACTION	NEUROTRANSMITTER	HYPOXIA SURVIVAL TIMES
PHYSOSTIGMINE	CENTRAL ACETYLCHOLINESTERASE INHIBITOR	↑ ACH	↑
NEOSTIGMINE	PERIPHERAL ACETYLCHOLINESTERASE INHIBITOR	↑ ACH	↔
GLYCOPYRROLATE + PHYSOSTIGMINE	PERIPHERAL CHOLINERGIC ANTAGONIST CENTRAL ACETYLCHOLINESTERASE INHIBITOR	↑ ACH	↑
ATROPINE SULFATE + PHYSOSTIGMINE	CENTRAL CHOLINERGIC ANTAGONIST CENTRAL ACETYLCHOLINESTERASE INHIBITOR	↑ ACH	↔

* SEE REFERENCE 21

Experimental Animals

Male C57BL/6J mice (Jackson Laboratory) 10 weeks old and weighing 25-30 grams were utilized in these experiments. The animals were housed (5 per cage) in stainless steel cages under a 12 hour light/dark (6 a.m.-6 p.m.) schedule at a temperature of 21 $\pm$ 2°C and relative humidity 30-60%. Standard (Purina Rodent Chow) food and water were available ad libidum.

Animals used in chronic studies received 1 mg/kg diisopropyl fluorophosphate-DFP (Aldrich Chemical Co.) or 10% propylene glycol dissolved in 0.9% saline (vehicle control animals). Injections were administered intraperitoneally in a volume of 0.01 ml/gram body weight between 7-9 a.m. Animals used in acute studies were injected intraperitoneally with a volume of 0.01 ml/gram body weight. In these acute studies mice dosed with 1 mg/kg DFP were compared to vehicle (10% propylene glycol dissolved in 0.9% saline) treated animals. In a second acute study, mice received 10 mg/kg atropine sulfate (Sigma Chemical Co.) or 0.9% saline which served as control.

Animal Sacrifice and Tissue Removal

The number of muscarinic receptors was assayed in brain homogenates taken from mice that were sacrificed by decapitation 24 hours after dosing except where otherwise indicated. Following decapitation, the brain tissue from each mouse was removed and the cerebellum was dissected away and discarded. The cerebrum was divided in half longitudionally and each cerebral hemisphere was placed in minivials and rapidly frozen in

a dry ice methanol bath (-72°C). The minivials were stored at -20°C for no more than one week.

Brains were assayed for specific [^{3}H]QNB (3-Quinuclidinyl benzilate) binding according to the method of Yamamura and Snyder (18) with minor modifications. [^{3}H]QNB (New England Nuclear, Specific activity 29.4-40.2 Ci/mmole), a specific muscarinic antagonist, was utilized to assess the affinity and number of muscarinic binding sites in cortical homogenates. One cerebral hemisphere from each mouse was placed in a 5 ml Potter Elvehjem glass homogenizer fitted with a Teflon pestle and were homogenized in 3 ml ice cold (4°C) 0.32 M sucrose for one minute at high speed (T-Line Laboratory stirrer motor). Tissue homogenates were then placed in a 10 ml borosilicate glass test tube and centrifuged for 10 minutes at 1,000 x g in an International PR-6000 centrifuge at 5°C. The pellet (crude nuclear fraction) was discarded and the supernatant (S1) containing crude synaptosomes was homogenized with the Brinkman Polytron (PCU-2-110, setting 5, for 30 seconds).

To assay for the total number of muscarinic receptors, a homogenate was incubated in 50 mM Na-K phosphate buffer pH 7.4 containing a concentration of 0.7 nM [^{3}H]QNB. The final incubation volume was 2 ml. Nonspecific binding of QNB to brain homogenate was determined in the presence of 1×10^{-6} M atropine sulfate. Atropine binds to all available specific muscarinic receptor sites in the tissue homogenate and therefore any [^{3}H]QNB bound to the tissue sample in the presence of atropine was defined as nonspecific binding.

Following an hour incubation at 25°C, the binding reaction was terminated by pouring the contents of each assay tube on a GF/B filter (Whatman). The [^{3}H]QNB bound to membrane protein remains on the filter, while free [^{3}H]QNB is washed away. Each filter was positioned over a manifold (Millipore Model 3205) filtering device under vacuum. The assay tube was washed four times with 4 ml ice cold 50 mM Na-K phosphate buffer pH 7.4 and each wash was applied to the filter. The filters were transferred to 20 ml polypropylene scintillation vials (Fisher Scientific) containing 9 ml J. T. Baker Uni/Verse scintillation cocktail. The vials were maintained at room temperature for 1-3 hours to allow counts to dissolve from the filters, vortexed and counted in a Beckman LS-9000 liquid scintillation spectrometer using a modified tritium program to determine disintegrations per minute.

A Scatchard analysis was utilized to determine the affinity and number of specific [^{3}H]QNB receptors in cortical homogenates (26).

Specific [^{3}H]QNB binding to tissue homogenates from different experimental groups was expressed as the mean of the group $\pm$ the standard error of the group. A Student-t analysis was utilized to determine the statistical significance between experimental groups. A p value $< .05$ between experimental groups was considered to be a significant difference.

Protein levels were determined by the method of Lowry et al. (27) using bovine serum albumin (Sigma Chemical Co.) as the standard.

Hypoxia Studies

A test chamber consisting of a clear plastic 22 x 21.5 x 20 cm. box with a cover was utilized for the hypoxia screen. Two minutes

prior to the test period a gas mixture containing 96% nitrogen and 4% oxygen (Union Carbide Corporation, Linde Division) was introduced into the test chamber through a valve near the bottom of the chamber and a valve at the top of the chamber was left open to allow the gases to escape. Gas pressure was maintained at 10 pounds per square inch with a Matheson model 8-590 regulator and flow was controlled with a Linde Model L32 flowmeter at 0.01 m^3/minute. Room temperature was maintained at 24 $\pm$ 2°C. The testing period began as five mice were placed into the screening chamber and the top valve was closed, while the gas mixture continued to flow into the screening chamber. Survival times of the mice to hypoxic conditions were assessed to the nearest one tenth of a minute. Survival time was recorded to a maximum of fifteen minutes. Student t was the statistical test ($p < .05$) was used to analyze acute and chronic alterations in hypoxia survival times. A two way analysis of variance (ANOVA) was the statistical test ($p < .05$) utilized for the analysis of survival times collected in the study of effect of time of day on hypoxia lethality. In that study survival times of mice were collected at 15 minute intervals during a two hour period on consecutive days.

Dosing and Tissue Sampling: Protocol for DFP Induced Subsensitivity

One hundred and fifty mice were dosed on Day 1. Mice were divided into two groups: seventy-five mice received DFP treatment, the remainder received drug vehicle injection every 72 hours following the first injection which was designated as Day 1. At various time intervals after the first injection of drug-vehicle or DFP, five mice from each treatment group were sacrificed and the number of central cholinergic

receptors was assayed utilizing a [^{3}H]QNB binding procedure. Figure 5 illustrates the dosing and sacrifice regimen. Following the first dose the number of central cholinergic receptors was assayed one and twenty four hours after the injection. Subsequent assays for the number of central cholinergic receptors were obtained at six day intervals following the first injection until the study was terminated on Day 65. DFP and vehicle control injections of mice were discontinued on Day 40.

Dosing and Tissue Sampling: Protocol for the Correlation of DFP Induced Subsensitivity and Hypoxia Survival Times

Two hundred mice were dosed according to the schedule that was used to induce cholineric subsensitivity. One hundred mice received drug treatment, the remainder received drug vehicle and served as controls. Twenty four hours after an injection on Days 5, 17, 35, 41 and 60, fifteen mice from each vehicle control and DFP-treated groups were tested utilizing the hypoxia test, and an additional five mice were sacrificed from each experimental group in order to determine central cholinergic receptor number (Figure 6). DFP and vehicle control injections were discontinued on Day 40.

FIGURE 5

Dosing and tissue sampling: protocol for DFP-induced cholinergic subsensitivity. C57BL/6J mice were dosed every 72 hours following the initial dose on Day 1 (▼). Mice were sacrificed and [^{3}H]QNB binding was assayed at various time points (↑).

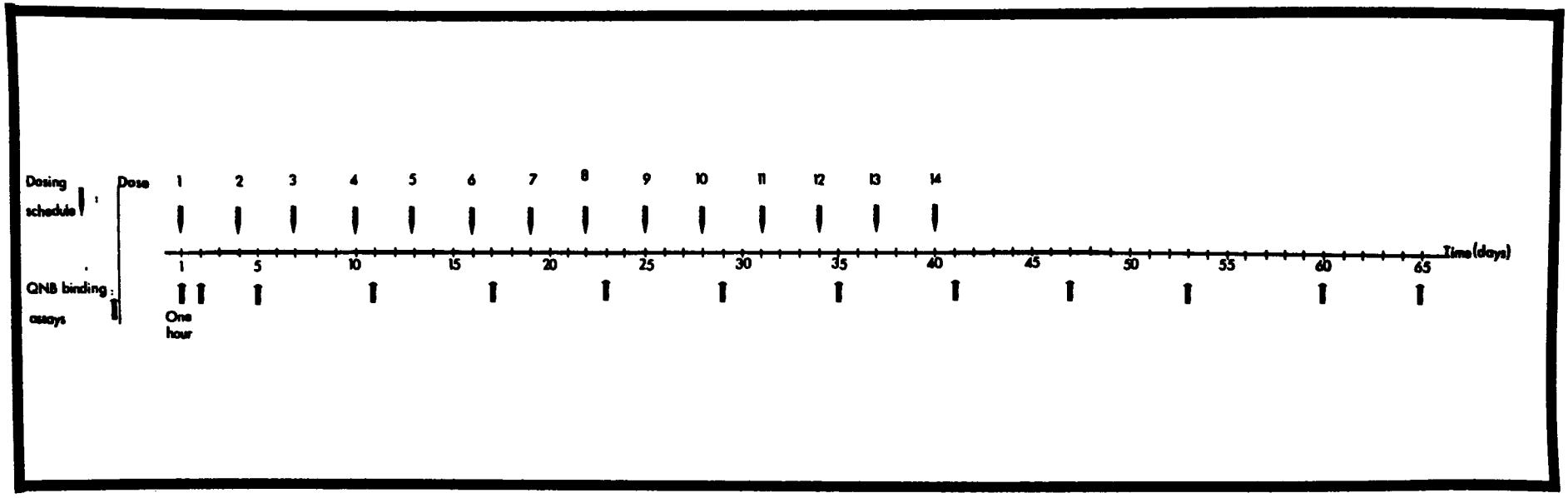
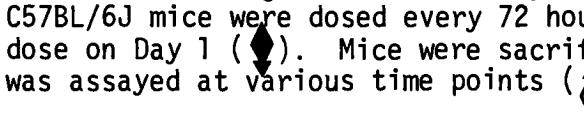
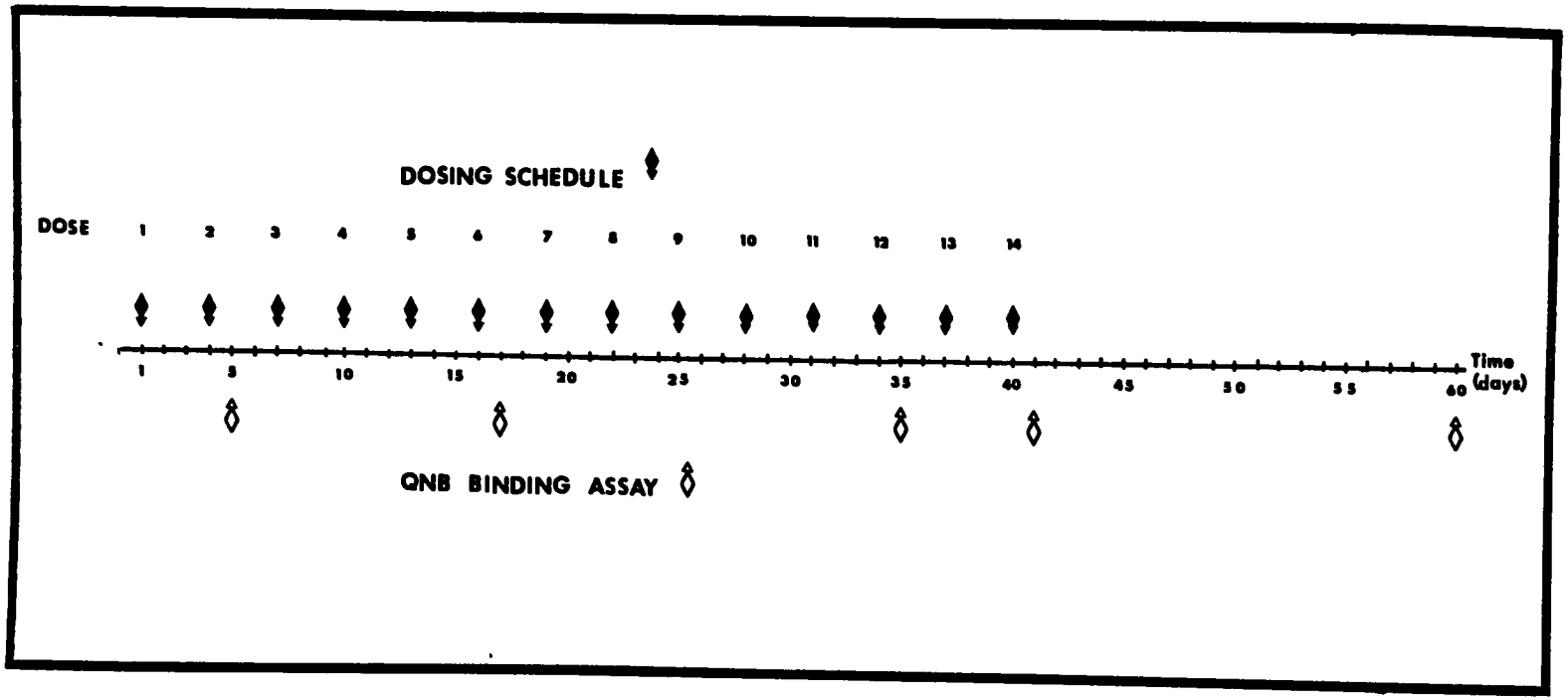

Dosing schedule
Dose
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)
1
5
10
15
20
25
30
35
40
45
50
55
60
65
QNB binding assays
One hour

FIGURE 6



Dosing and tissue sampling: Protocol for the correlation of DFP-induced cholinergic subsensitivity and hypoxia survival times. C57BL/6J mice were dosed every 72 hours following the initial dose on Day 1 (◆). Mice were sacrificed and [^{3}H]QNB binding was assayed at various time points (◇).


DOSING SCHEDULE
DOSE
1
2
3
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25
30
35
40
45
50
55
60
Time (days)
QNB BINDING ASSAY

RESULTS

Preliminary Hypoxia Screen Studies

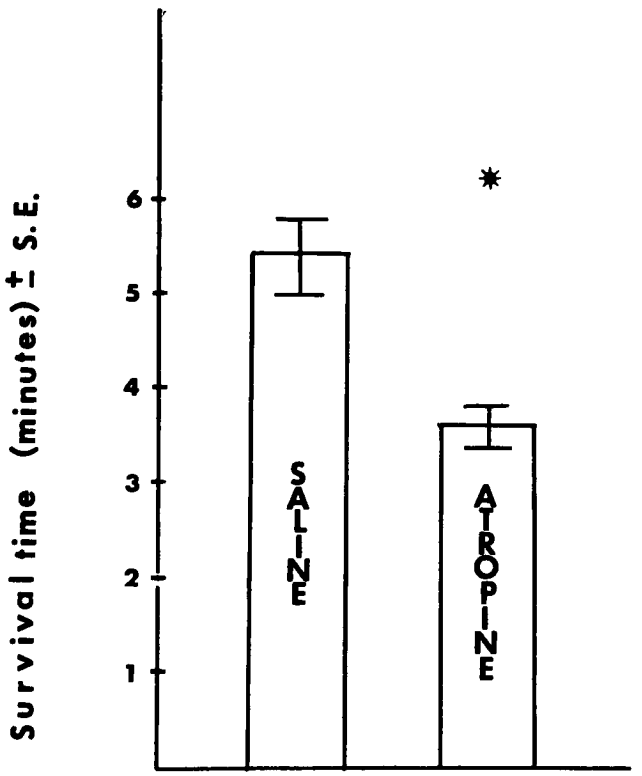
Exposure of mice to hypoxic conditions generally produced the following series of changes that ultimately led to respiratory failure. Mice placed in the hypoxia chamber remained relatively stationary until several minutes into the testing period, then they began to explore the chamber until they lost their righting reflex. At this point obvious signs of cyanosis, writhing and gasping were evident. The final moments before respiratory failure were characterized by spontaneous muscular contraction and intermittent gasping. Survival times under hypoxic conditions were recorded at the last respiratory movement.

These experiments were designed to examine the effect of acute administration of cholinergic drugs on hypoxia survival times in mice. Figure 7 shows the survival time of mice injected with atropine (10 mg/kg) or saline intraperitoneally (i.p.) 30 minutes prior to exposure to hypoxic conditions. The mean survival time of control mice was 5.4 $\pm$ 0.4 minutes while the mean survival time for atropine treated mice was 3.6 $\pm$ 0.2 minutes. Intraperitoneal injections of 10 mg/kg atropine sulfate in mice caused a significant decrease in survival time of mice exposed to hypoxic conditions compared to saline-injected control animals (Student t $p < .05$). This dose of atropine did not produce any overt behavioral changes.

FIGURE 7

Hypoxia survival time of C57BL/6J mice (n=10) injected with 0.9% saline or 10 mg/kg atropine sulfate injected intraperitoneally in mice 30 minutes prior to exposure to hypoxic conditions.

* Significant $p < .05$, Student t test.


Survival time (minutes) ± S.E.
6
5
4
3
2
1
SALINE
ATROPINE
*

The effect of 1 mg/kg DFP i.p. administration in mice on hypoxia survival times was examined in the next experiment. Injections of DFP into mice 30 minutes prior to exposure to hypoxia significantly increased survival time compared to vehicle-control mice (Student t $p < .05$). Figure 8 shows the survival times of vehicle-control mice was 6.9 ± 0.4 minutes while DFP treated mice survived an average of 9.8 ± 0.8 minutes. No cholinergic symptomology was evident at this dose of DFP.

The data summarized in the acute atropine and DFP studies were collected over a two day period. On each day, five control mice were tested in the hypoxia chamber and five drug treated mice were tested immediately thereafter. Data were pooled to obtain n=10 for each experimental group.

Studies have shown that survival times vary with time of day, therefore the next experiments were designed to study the variations of survival times during the time intervals of the day that would be used in future studies. A group of 100 nontreated mice were divided into two experimental groups and studied on successive days. Testing began at 7:45 a.m. and survival times were assessed at fifteen minute intervals until 10 a.m. Results are summarized in Table 2. There were no significant variations in hypoxia survival times (ANOVA, $p < .05$) during the time intervals studied. Hypoxia survival times followed a normal distribution (Figure 9). Hypoxia survival times recorded during the two hour period on Day 1 yielded a mean survival time of 5.4 ± 0.1 minutes with a range of 3.4-7.4 minutes, on Day 2 the mean survival time was 5.5 ± 0.1, range 4.0-7.4 minutes. All subsequent hypoxia testing was scheduled during this time interval.

FIGURE 8

Survival time of C57BL/6J mice (n=10) with 10% propylene-glycol dissolved in 0.9% saline or 1 mg/kg DFP injected intraperitoneally in mice 30 minutes prior to exposure to hypoxic conditions.

* Significant $p < .05$, Student t test.

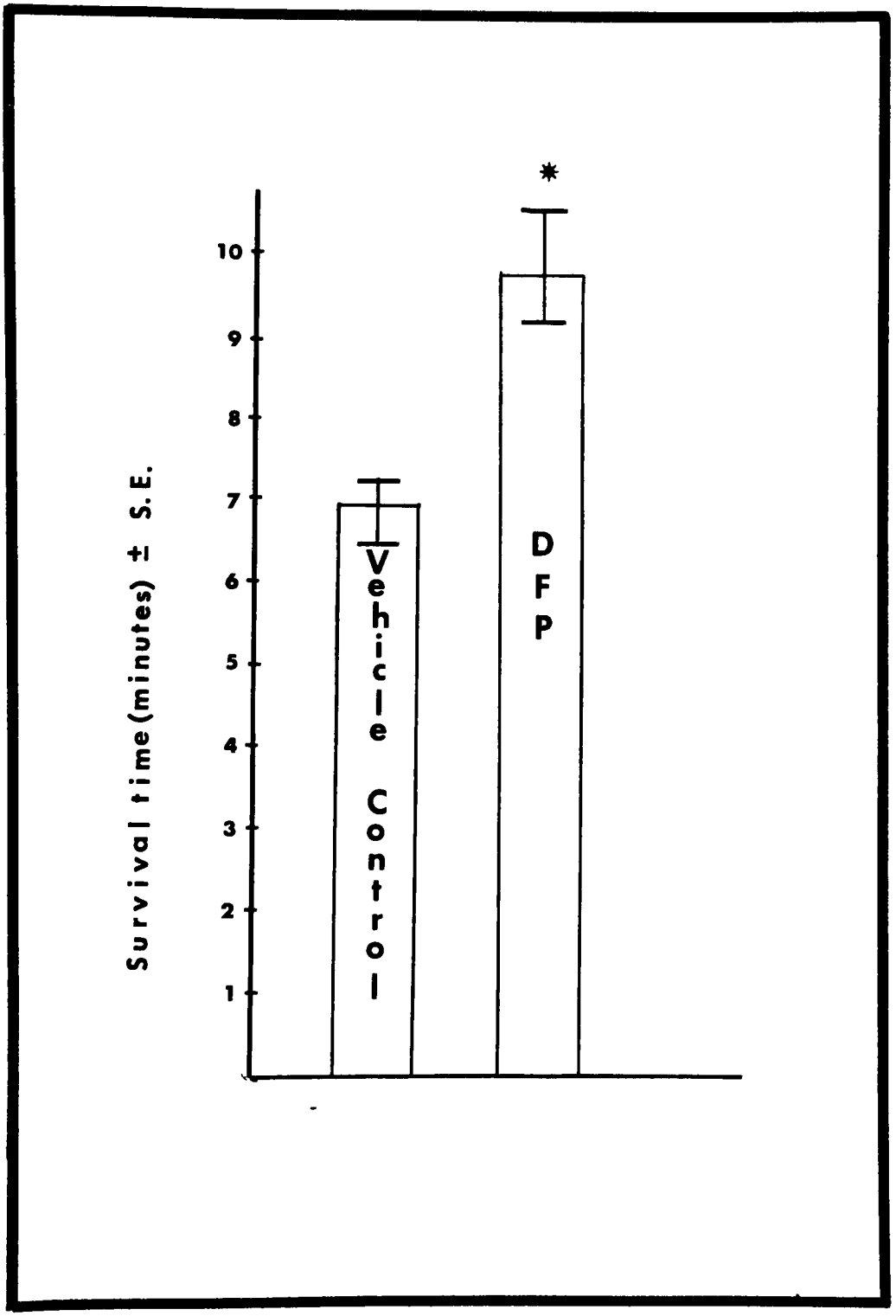
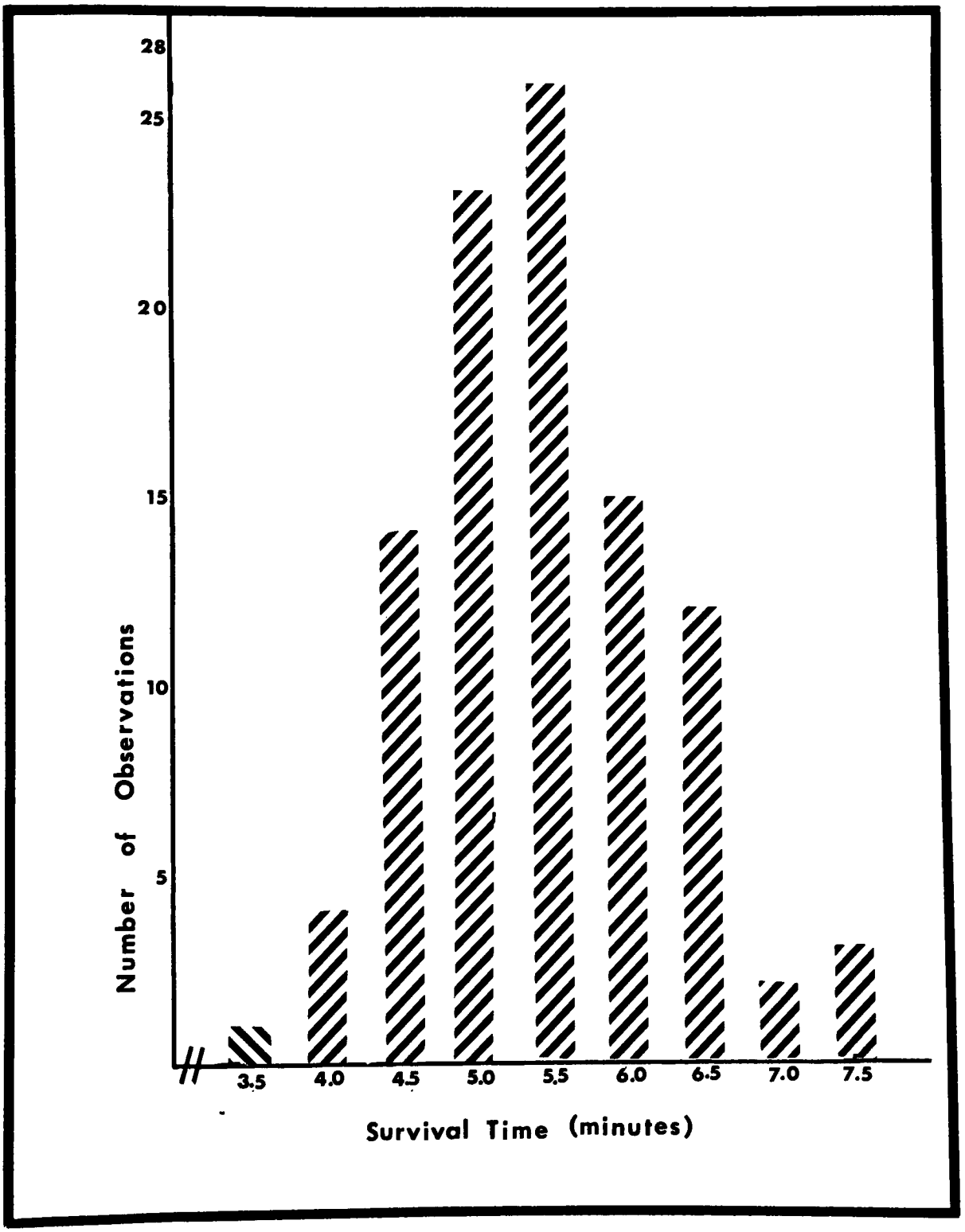

Survival time (minutes) ± S.E.
10
9
8
7
6
5
4
3
2
1
*
Vehicle Control
DFP

TABLE 2: HYPOXIA LETHALITY IN C57BL/6J MICE AND TIME OF DAY

TIME	DAY	N	SURVIVAL TIMES (MINUTES)	
A.M.			RANGE	MEAN ± S.E.
7:45	1	5	4.1-6.5	5.6 ± 0.5
	2	5	4.7-6.5	5.6 ± 0.3
8:00	1	5	4.6-5.7	5.1 ± 0.2
	2	5	4.5-6.8	5.5 ± 0.4
8:15	1	5	4.2-5.6	5.0 ± 0.3
	2	5	4.0-6.1	5.2 ± 0.3
8:30	1	5	3.4-7.3	5.3 ± 0.7
	2	5	4.3-6.0	5.3 ± 0.3
8:45	1	5	4.8-6.6	5.5 ± 0.3
	2	5	4.7-6.0	5.1 ± 0.2
9:00	1	5	4.7-7.4	5.9 ± 0.5
	2	5	5.8-6.5	6.1 ± 0.4
9:15	1	5	5.3-6.2	5.7 ± 0.2
	2	5	5.0-7.4	6.0 ± 0.4
9:30	1	5	4.4-5.5	5.1 ± 0.4
	2	5	5.1-5.4	5.2 ± 0.1
9:45	1	5	4.7-6.7	5.7 ± 0.3
	2	5	5.1-6.4	5.8 ± 0.2
10:00	1	5	4.0-6.1	5.2 ± 0.3
	2	5	5.0-6.9	5.5 ± 0.4

FIGURE 9

Histogram of hypoxia survival times of 100 nontreated C57BL/6J mice observed on two consecutive days from 7:45 to 10:00 a.m. showing an approximate normal distribution. The number of observations (y-axis) are presented for survival times with the midpoint of each range of survival time (x-axis).


28
25
20
15
10
5
Number of Observations
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
Survival Time (minutes)

Preliminary [^{3}H]QNB Binding Studies

A Scatchard analysis of [^{3}H]QNB binding to cortical homogenates was used to determine the number and affinity of central muscarinic receptors. [^{3}H]QNB binding to cortical muscarinic receptors was measured at seven concentrations of radioligand. The Scatchard plot of [^{3}H]QNB binding to the muscarinic receptor in cortical homogenates of C57BL/6J mice resulted in a straight line which suggested a single species of independent receptors. The number of binding sites is 1.26 pmoles/mg protein with affinity of 0.27 nM (Table 3, Figure 10).

In order to assess the number of cholinergic receptors in mouse cortical homogenates, varying amounts of [^{3}H]QNB ligand were added to the incubation mixture to determine ligand concentration to be used in further binding studies (Table 4, Figure 11). Specific [^{3}H]QNB binding in mouse cortical homogenates was saturable with increasing concentrations of QNB, while nonspecific binding, in the presence of 1×10^{-6} M atropine sulfate, was not saturable and increased linearly with increasing concentration of [^{3}H]QNB. To measure the total number of muscarinic receptors in cortical homogenates a final concentration of 0.7 nM was selected. At this concentration less that 2% of [^{3}H]QNB was bound nonspecifically and less than 30% of the total concentration of [^{3}H]QNB was bound to muscarinic receptors in mouse cortical homogenates.

Linearity of [^{3}H]QNB binding to cortical homogenates was checked using a range of protein concentrations varying from 0.1 to 1.0 mg protein/assay. Total and nonspecific binding were measured at various

TABLE 3: SCATCHARD PLOT-[^{3}H]QNB BINDING TO MUSCARINIC RECEPTORS IN C57BL/6J MOUSE CORTICAL HOMOGENATES

[^{3}H]QNB INPUT (FMOLE)	[^{3}H]QNB FREE (FMOLE/nM)	[^{3}H]QNB BOUND (PMOLE/MG)	[^{3}H]QNB BOUND/FREE $\frac{\text{(PMOLE/MG)}}{\text{nM}}$
68	0.02	0.07	4.32
957	0.32	0.70	2.21
1287	0.46	0.81	1.79
2675	1.10	1.03	0.93
6759	3.12	1.12	0.36
9969	4.72	1.16	0.25
14050	6.76	1.23	0.18

FIGURE 10

Scatchard plot of [^{3}H]QNB binding to muscarinic receptors in C57BL/6J mouse cortical homogenates. [^{3}H]QNB bound in pmole/mg protein (x-axis) vs. [^{3}H]QNB Bound/Free (y-axis) in (pmole/mg protein)/nM. The number of binding sites B_{max} is 1.26 pmole/mg protein with affinity K_d of 0.27 nM. The slope of the line obtained from linear regression analysis is -3.70.

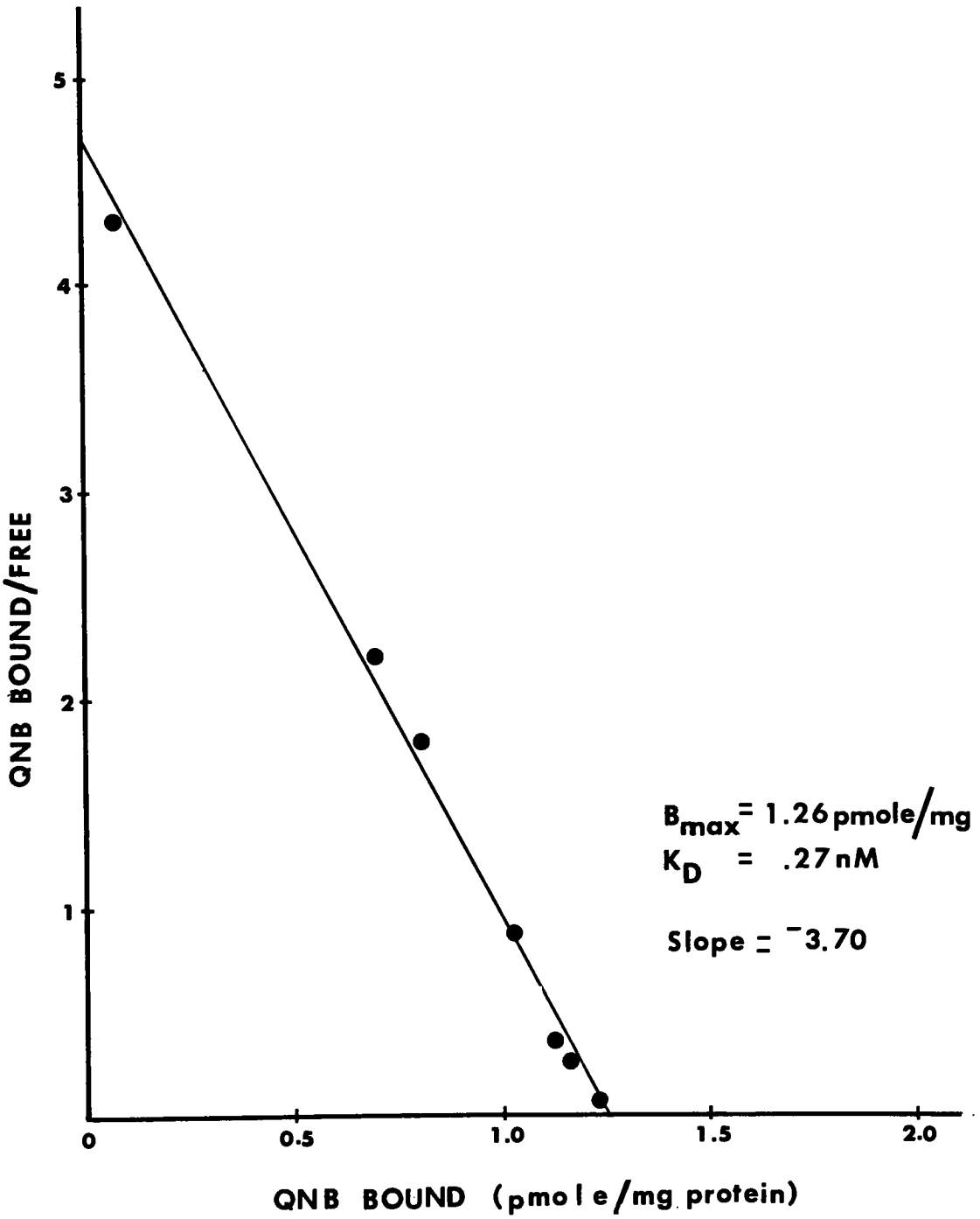
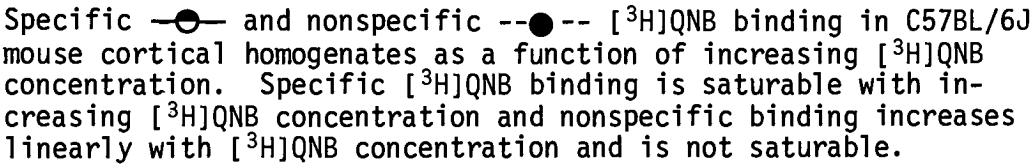
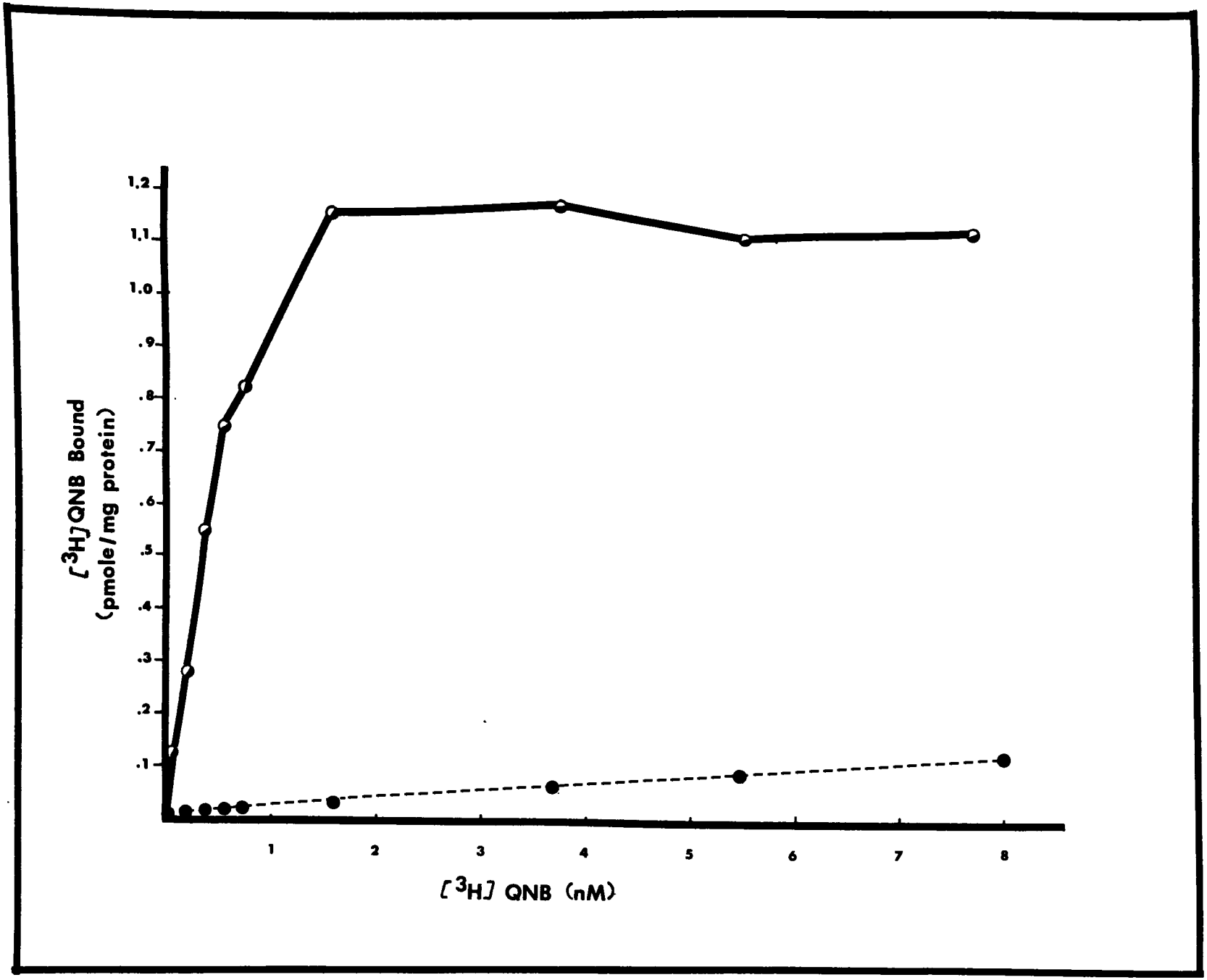

5
4
3
2
1
0
0.5
1.0
1.5
2.0
QNB BOUND/FREE
QNB BOUND (pmole/mg protein)
B_{max} = 1.26 pmole/mg
K_D = .27 nM
Slope = ⁻3.70

TABLE 4: SPECIFIC BINDING OF [^{3}H]QNB TO C57BL/6J MOUSE CORTICAL HOMOGENATES AS A FUNCTION OF [^{3}H]QNB CONCENTRATION

[^{3}H]QNB (nM)	[^{3}H]QNB BOUND (PMOLE/MG PROTEIN)	
	SPECIFIC	NONSPECIFIC
0.08	0.12	0.01
0.18	0.28	0.01
0.37	0.55	0.01
0.56	0.75	0.01
0.72	0.83	0.01
1.58	1.15	0.03
3.73	1.17	0.05
5.44	1.12	0.08
7.57	1.13	0.13

FIGURE 11


Specific —⊖— and nonspecific --●-- [3H]QNB binding in C57BL/6J mouse cortical homogenates as a function of increasing [3H]QNB concentration. Specific [3H]QNB binding is saturable with increasing [3H]QNB concentration and nonspecific binding increases linearly with [3H]QNB concentration and is not saturable.


[3H]QNB Bound
(pmole/mg protein)
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
1
2
3
4
5
6
7
8
[3H] QNB (nM)

concentrations of protein. Specific [^{3}H]QNB binding was linear between 0.1 and 0.5 mg of tissue protein (Table 5, Figure 12). The protein concentration used in subsequent assays varied between 0.4-0.45 mg protein/assay tube.

[^{3}H]QNB Binding Studies: DFP-Induced Central Cholinergic Subsensitivity

This study was designed to examine the time course of development and recovery of DFP-induced central cholinergic subsensitivity in C57BL/6J mice. Table 6 and Figure 13 summarize the results of this study. The 17th day of treatment (6th dose of DFP) was the first time point at which there was a significant difference in the number of central cholinergic receptors in vehicle-treated animals compared to DFP-treated animals. Maximal DFP-induced cholinergic subsensitivity was detected by the 23rd day of drug treatment and remained constant until after the 41st day of the experiment. At these time points QNB binding to cortical muscarinic receptors of DFP-treated mice was decreased by 20-25% compared to vehicle control animals. The levels of [^{3}H]QNB binding to central muscarinic receptors in cortical homogenates of DFP-treated animals continued to be significantly reduced when compared to vehicle control animals 7 days (Day 47) after the last injection. The number of central cholinergic receptors was 15% less in DFP-treated animals compared to vehicle control mice at this time. The number of cholinergic receptors at this time point was significantly greater than QNB binding values on Days 23 through 41. Thirteen days (Day 53) after the final injection of DFP the level of [^{3}H] QNB binding in cortical homogenates obtained from DFP-treated mice was not significantly different than the binding levels in vehicle control mice.

TABLE 5: SPECIFIC BINDING OF [^{3}H]QNB AS A FUNCTION OF TISSUE PROTEIN CONCENTRATION

MG PROTEIN	MEAN DPM ± S.E. TOTAL	NONSPECIFIC	SPECIFIC DPM	PMOLE [^{3}H]QNB BOUND
0.196	10556 ± 213	548 ± 45	10008	0.155
0.245	14330 ± 332	555 ± 48	13775	0.213
0.492	27270 ± 117	618 ± 78	26652	0.412
0.738	31649 ± 950	617 ± 14	31032	0.480
0.984	34598 ± 171	718 ± 31	33880	0.524

FIGURE 12

Results of specific [^{3}H]QNB binding to muscarinic receptors in C57BL/6J mouse cortical homogenate as a function of protein concentration. Specific [^{3}H]QNB binding was linear between 0.1 and 0.5 mg of tissue protein.

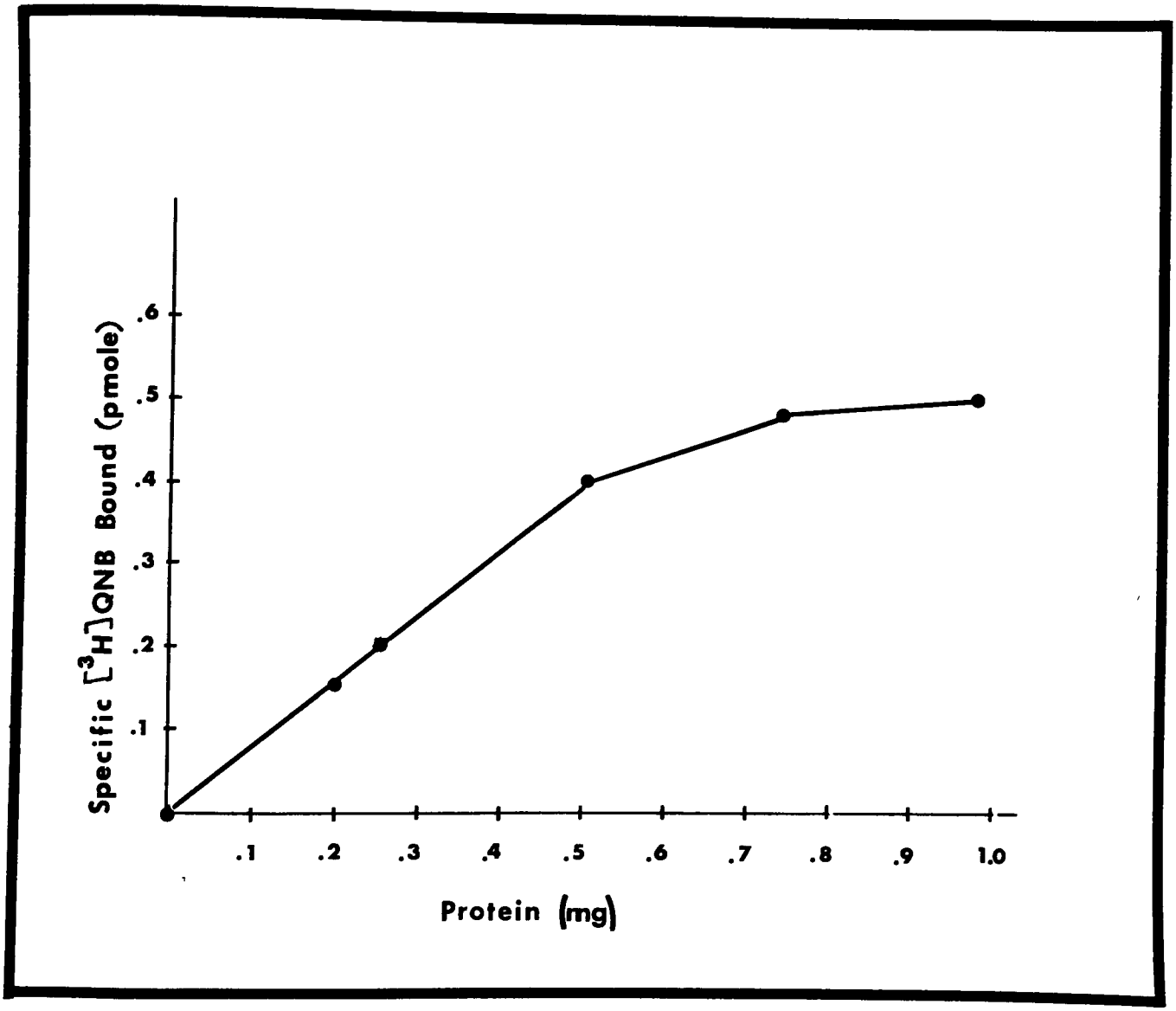

Specific [3H]QNB Bound (pmole)
.6
.5
.4
.3
.2
.1
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Protein (mg)

TABLE 6: TIME COURSE OF DEVELOPMENT AND RECOVERY OF DFP-INDUCED CENTRAL CHOLINERGIC SUBSENSITIVITY OF C57BL/6J MICE

		SPECIFIC BINDING OF [^{3}H]QNB			
DAY	DOSE # OF DFP	CONTROL (PMOLE/MG)	DFP (PMOLE/MG)	% CONTROL	
1	1	1.132 ± 0.038	1.125 ± 0.027	99.4 ± 2.4	SACRIFICED ONE HOUR POST DOSE
2	1	1.143 ± 0.041	1.152 ± 0.050	100.8 ± 4.4	SACRIFICED 24 HOURS POST DOSE
5	2	0.986 ± 0.012	0.971 ± 0.008	97.9 ± 0.6	"
11	4	0.805 ± 0.036	0.725 ± 0.023	90.1 ± 2.8	"
17	6	0.781 ± 0.016	0.687 ± 0.018	88.4 ± 2.4*	"
23	8	0.892 ± 0.028	0.708 ± 0.016	79.4 ± 1.8*	"
29	10	0.886 ± 0.040	0.676 ± 0.016	76.3 ± 1.8*	"
35	12	0.909 ± 0.020	0.686 ± 0.032	75.4 ± 3.5*	"
41	14	0.911 ± 0.010	0.725 ± 0.010	79.6 ± 1.1*	SACRIFICED 24 HOURS AFTER THE LAST DOSE OF DFP
47	6 DAYS POST FINAL DOSE OF DFP	0.980 ± 0.023	0.835 ± 0.013	85.2 ± 1.3*	
53	13 DAYS POST FINAL DOSE OF DFP	0.942 ± 0.007	0.934 ± 0.019	99.1 ± 2.0	
60	20 DAYS POST FINAL DOSE OF DFP	1.043 ± 0.019	1.045 ± 0.032	100.6 ± 3.1	
65	25 DAYS POST FINAL DOSE OF DFP	1.080 ± 0.062	1.112 ± 0.041	103.4 ± 4.1	

* SIGNIFICANT STUDENT T TEST $P<.05$

FIGURE 13

Results of time course of development and recovery of DFP-induced central cholinergic subsensitivity in C57BL/6J mice (n=5).

✱ Significant $p < .05$ Student t test.

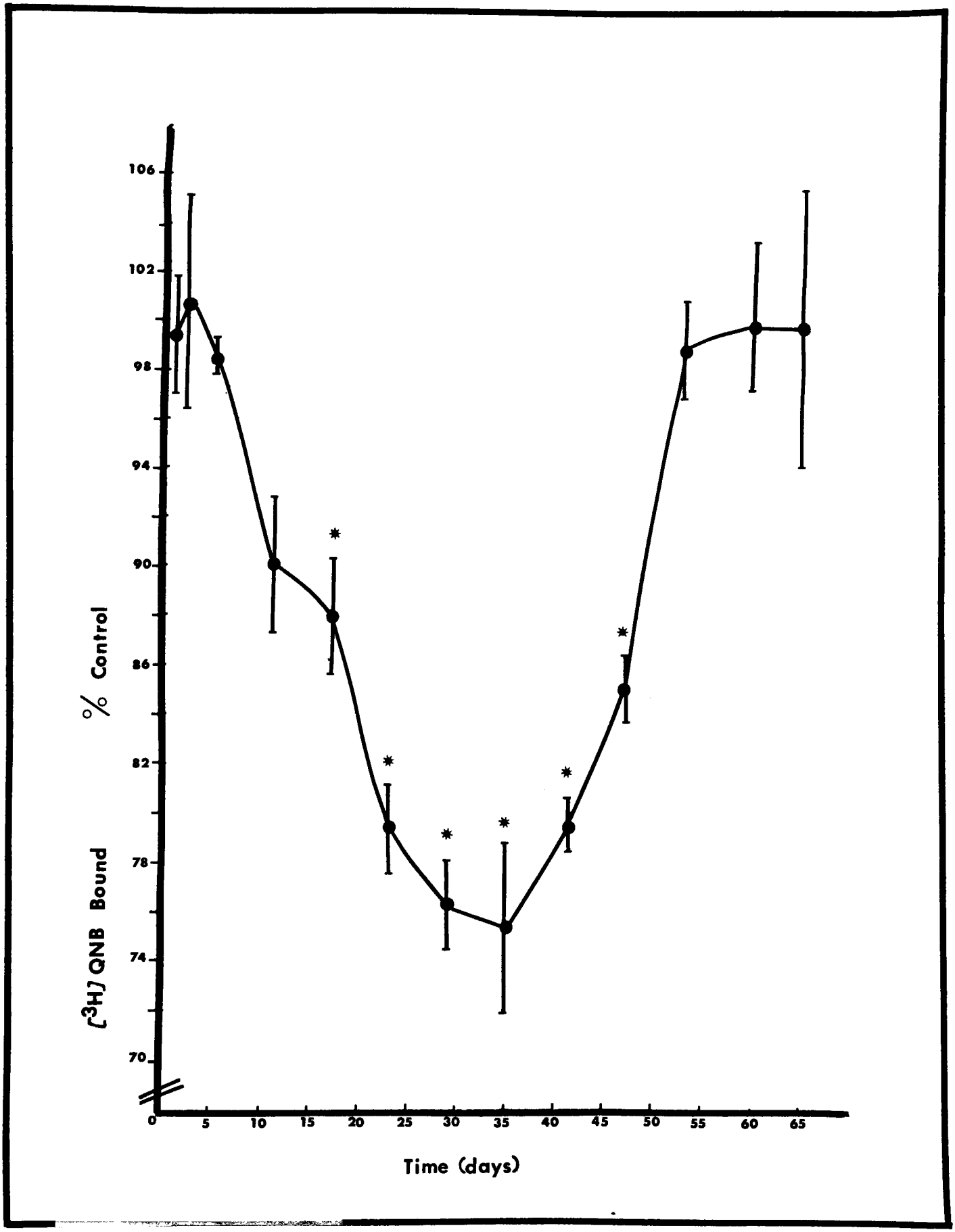

106
102
98
94
90
86
82
78
74
70
% Control
[3H] QNB Bound
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (days)

Table 6 also illustrates [^{3}H]QNB binding to cortical muscarinic receptors in vehicle control mice showed significant alterations in receptor binding levels compared to vehicle control at subsequent time points. Receptor binding levels in cortical homogenates of vehicle control animals on Day 11-53 were significantly depressed with the exception of Day 47 compared to vehicle control animals on Day 1.

The next experiment was designed to: (a) determine if survival times of mice exposed to hypoxic conditions could be altered by chronic DFP administration and (b) determine if there is a correlation between the number of central cholinergic receptors and survival times to hypoxia.

Results from the [^{3}H]QNB receptor binding assay are illustrated in Table 7 and Figure 14 Panel A; the hypoxia survival time results are shown in Table 8 and Figure 14 Panel B. On Day 5, after two doses of DFP, QNB binding to central muscarinic receptor sites was not altered, but hypoxia survival times of DFP-treated mice (6.7 $\pm$ 0.5 minutes) were significantly increased compared to vehicle control mice (5.4 $\pm$ 0.2 minutes). The 17th day of treatment (dose 6) was the first time point at which there was a significant difference between the level of central cholinergic receptor binding in cortical homogenates of DFP-treated mice as compared to vehicle control animals. Receptor numbers in the DFP treated mice were 86% of vehicle control levels on Day 17 (significant Student t $p < .05$). Survival time of DFP-treated mice in the hypoxia test (7.1 $\pm$ 0.3 minutes) was significantly increased compared to vehicle control mice (5.5 $\pm$ 0.2 minutes) at this same time point. Maximal DFP-induced subsensitivity of muscarinic

TABLE 7: CORRELATION STUDY: TIME COURSE OF DEVELOPMENT AND RECOVERY OF DFP-INDUCED CHOLINERGIC SUBSENSITIVITY IN C57BL/6J MICE

DAY	DOSE # OF DFP	SPECIFIC [^{3}H]QNB BINDING		
		CONTROL (PMOLE/MG)	DFP (PMOLE/MG)	% CONTROL
5	2	0.806 ± 0.011	0.794 ± 0.034	98.5 ± 4.2
17	6	0.824 ± 0.028	0.713 ± 0.018	86.6 ± 2.2 *
35	12	0.854 ± 0.042	0.609 ± 0.027	71.3 ± 3.1 *
41	14	0.767 ± 0.020	0.541 ± 0.065	68.7 ± 1.4 *
60	20 DAYS POST FINAL DFP DOSE	0.748 ± 0.014	0.665 ± 0.023	88.2 ± 3.0 *

* SIGNIFICANT STUDENT T TEST $P < .05$

TABLE 8 : CORRELATION STUDY: HYPOXIA SURVIVAL TIMES DURING THE TIME COURSE OF DEVELOPMENT AND RECOVERY OF DFP-INDUCED CENTRAL CHOLINERGIC SUBSENSITIVITY OF C57BL/6J

DAY	DOSE # OF DFP	N	MEAN SURVIVAL TIME ± S.E. MINUTES		
			CONTROL	DFP	
5	2	15	5.4 ± 0.2	6.7 ± 0.5	*
17	6	15	5.5 ± 0.2	7.1 ± 0.3	*
35	12	15	5.3 ± 0.2	10.3 ± 0.7	*
41	14	15	5.0 ± 0.4	8.5 ± 0.8	*
60	20 DAYS POST FINAL DOSE (#14) OF DFP	15	5.1 ± 0.2	6.1 ± 0.5	

* SIGNIFICANT STUDENT T TEST $P<.05$

FIGURE 14

Correlation Study: Time course of development and recovery of DFP-induced central cholinergic subsensitivity in C57BL/6J mice and hypoxia survival times.

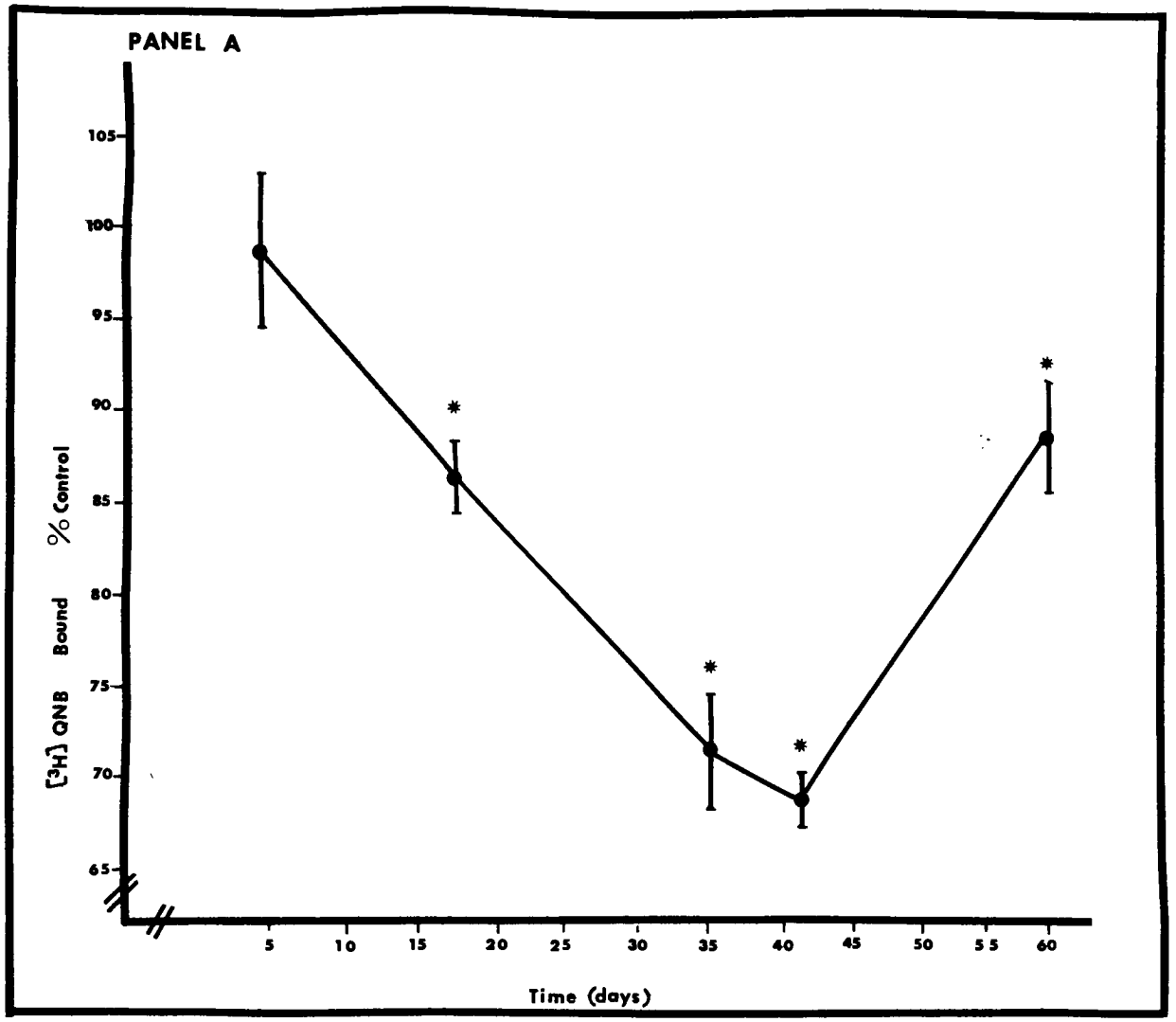
PANEL A

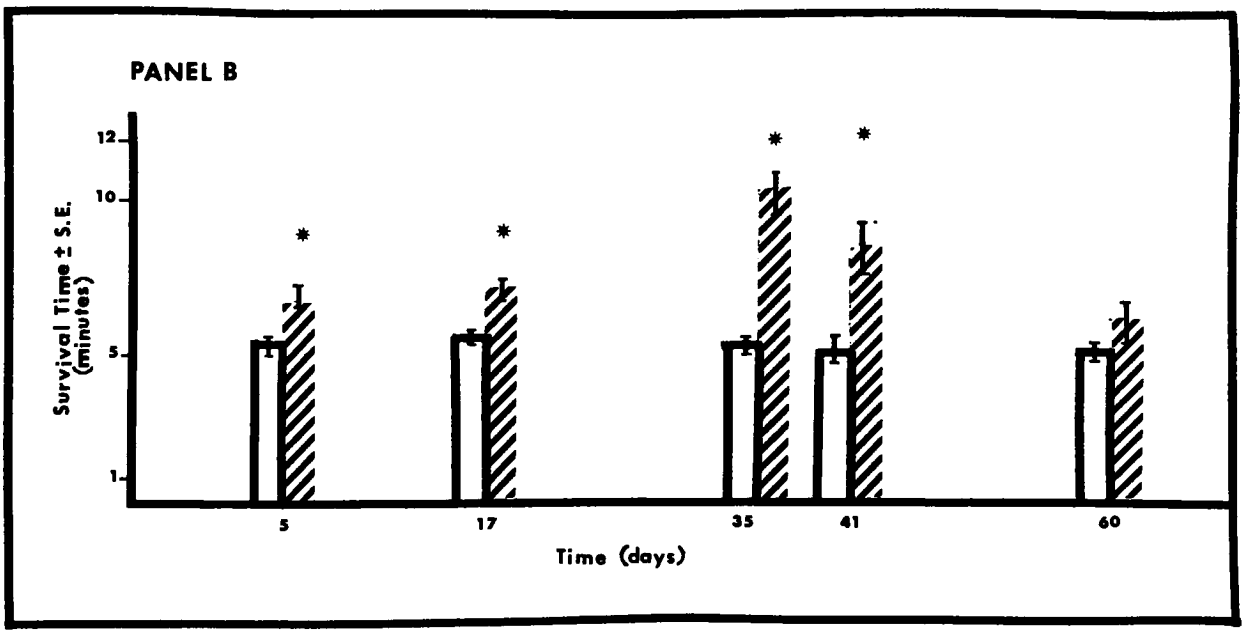
Results of time course of development and recovery of DFP-induced central cholinergic subsensitivity in C57BL/6J mice (n=5).

PANEL B

Results of hypoxia survival times of C57BL/6J mice (n=5) during time course of development and recovery of DFP-induced central cholinergic subsensitivity.

* Significant $p < .05$ Student t test.


PANEL A
105
100
95
90
85
80
75
70
65
[3H] QNB Bound % Control
5
10
15
20
25
30
35
40
45
50
55
60
Time (days)


PANEL B
12
10
5
1
Survival Time ± S.E. (minutes)
5
17
35
41
60
Time (days)

receptors was measured after the 12th dose (Day 35) and the 14th dose of DFP (Day 41). A comparison of the number of receptors in cortical homogenates of DFP-treated mice on these days showed no statistical difference between receptor levels in drug treated mice. At these time points QNB binding to central muscarinic receptors was decreased by 25-30% in DFP-treated mice when compared to QNB binding levels in cortical homogenates of vehicle control mice. Hypoxia survival times in DFP treated mice were significantly increased as compared to control vehicle mice after the 12th (Day 35) and 14th doses (Day 41) of DFP. By the 20th day after DFP withdrawal (Day 60) the number of central cholinergic receptors in the cortical homogenates of DFP-treated mice rose to 86% of the number of these receptors in vehicle control mice (significant Student t $p < .05$). At this same time point, 20 days (Day 60) after drug withdrawal, there was no statistically significant alteration in the hypoxia survival times of DFP-treated mice compared to vehicle control survival times. Mean survival of DFP-treated mice was 6.1 ± 0.5 minutes and vehicle control mice survived an average of 5.1 ± 0.2 minutes in the hypoxia test on Day 60.

DISCUSSION

Numerous studies have demonstrated that survival time of experimental animals to hypoxic conditions can be altered by manipulation of the central cholinergic nervous system (21-23). This type of screen has been utilized to assess the effect of drugs on cholinergic neurons. Scremin (21-22) has shown that drugs which enhance central cholinergic activity increase hypoxia survival time, whereas drugs that reduce central cholinergic activity reduce survival time. These studies also demonstrate that this effect is not mediated by peripheral cholinergic receptors (22).

Our initial study was designed to determine if C57BL/6J mice were an appropriate animal model to study the effects of centrally acting cholinergic drugs on hypoxia survival times. A single intraperitoneal injection of atropine sulfate in C57BL/6J mice was shown to decrease hypoxia survival time by 39% when compared to the appropriate control group. These results are similar to Scremin's (21) who demonstrated that atropine sulfate decreased survival time by 33% in mice. In contrast to the effect of atropine sulfate on hypoxia survival times, a single intraperitoneal dose of DFP increased the survival time of C57BL/6J mice to hypoxic conditions by 30%. Scremin and Atru (21-23) reported similar results for another acetylcholinesterase inhibitor, physostigmine, in mice. Our results demonstrated that hypoxia survival times of C57BL/6J were sensitive to acute alterations in central cholinergic activity.

Hypoxia survival times were obtained during a two hour period in the morning during which all subsequent experiments were performed. Reports have suggested that hypoxia survival times and cholinergic activity vary with time of day (28). Perry also reported that circadian rhythms alter central cholinergic activity in man (29). Survival times of C57BL/6J mice were assessed from 7:45 to 10:00 a.m. During this two hour period, survival times of untreated C57BL/6J mice remained stable.

It has been well established that chronic treatment of experimental animals with DFP creates a cholinergic subsensitivity i.e. reduced number of muscarinic receptors in the central nervous system (17-20). Chronic administration of DFP has been shown to reduce the number but not the affinity of the postsynaptic muscarinic receptor to cholinergic antagonists (18). A QNB binding assay (16) was used to measure the number and affinity of central cholinergic receptors. The mechanism by which chronic DFP administration induces a reduction in the number of central cholinergic receptors is thought to be due to acetylcholinesterase inhibition and the accumulation of acetylcholine at cholinergic receptors (18,20).

Cholinergic subsensitivity has been reported to be evident to the cerebral cortex of Alzheimer or senile dementia patients (7-9). If artificially induced cholinergic subsensitivity could be created by chronic DFP treatment, a suitable animal model may be developed which can be utilized to screen drugs for potential pharmacological activity in the treatment of certain aspects of senile dementia.

The [^{3}H]QNB binding procedure was modified to measure the number and affinity of central cholinergic receptors in C57BL/6J mice.

[^{3}H]QNB binding to the muscarinic receptor in mouse cerebral cortex was linear in the range of tissue protein from 0.1 to 0.5 mg, which agree well with previously published rodent data (16). The affinity of the receptor, K_d, is 0.27 nM and the number of muscarinic binding sites B_{max} is 1.26 pmoles/mg, which are comparable to published values for [^{3}H]QNB binding in rodent cerebral cortex (16,18). The number of muscarinic receptors in mouse cortical homogenates was determined with a concentration of 0.7 nM [^{3}H]QNB and was expressed as specific binding (pmole/mg). At this concentration, maximal binding and a high ratio of specific to nonspecific binding was obtained.

The onset and recovery of alterations in the number of central muscarinic receptors were characterized in C57BL/6J mice chronically treated with DFP. The maximal reduction in muscarinic receptor binding to QNB in cerebral cortex of DFP-treated mice was 70-75% of control animals, comparable to results previously reported (19). Cortical muscarinic receptor binding to QNB gradually decreased with time under a dose regimen of 1 mg/kg DFP administered every 72 hours for a total of 14 doses (40 days). The first significant difference in the number of central cholinergic receptors in DFP and vehicle control mice occurred after the 6th dose of DFP (Day 17) with maximal reduction evident after the 8th dose of DFP (Day 23). Receptor binding levels gradually returned to control levels 13 days (Day 53) after the final dose of DFP. Upon termination of DFP administration, acetylcholinesterase inactivated by DFP was replaced by newly synthesized acetylcholinesterase which was able to hydrolyze acetylcholine and reduced its' accumulation in the central synapse. The formation of new acetylcholinesterase reduced

hyperstimulation of central cholinergic neurons and this was thought to stimulate the synthesis of new receptors, which was reflected in increased [^{3}H]QNB binding. Our data provides the first information on the time course of replacement of central cholinergic receptors following withdrawal of chronic DFP administration. The results of this study indicated that at least 13 days are required for the number of central muscarinic receptors to return to control levels following chronic DFP administration. This study also showed that chronic administration of DFP may produce an "artificial form" of senility that is reflected in a cholinergic subsensitivity similar to that seen in Alzheimer's disease.

Next, we wanted to determine if these changes in cortical receptor binding levels could be detected by utilizing the hypoxia screen. If DFP-induced subsensitivity is to be utilized as a screening model, detection of small changes in central cholinergic activity could be simplified by monitoring these changes through the hypoxia screen.

Because our studies demonstrated that chronic administration of DFP could reversibly alter the number of central cholinergic receptors, we then examined the relationship between hypoxia survival times and [^{3}H]QNB binding to central cholinergic receptors. Presently, there are no studies which report any relationship between DFP-induced changes in receptor numbers and hypoxia survival times. Our results show that after the 6th (Day 17), 12th (Day 35), and 14th (Day 41) dose of DFP the survival times of C57BL/6J mice to hypoxia were significantly increased, while muscarinic receptor binding levels were significantly reduced when compared to vehicle control mice. The increase in

survival times on Days 5-41 (Dose 2-14) can possible be attributed to acetylcholine which accumulated in the synapse and a concomitant hyperstimulation of available acetylcholine receptor sites.

In contrast to this inverse relationship between hypoxia survival times and cholinergic receptor number, hypoxia survival times were significantly increased after the second dose of DFP (Day 5), but receptor number remained unaltered. In addition, hypoxia survival times in mice in which DFP administration had been terminated 20 days prior to testing on Day 60, were not significantly altered although there was a significant decrease in the number of central cholinergic receptors. The lack of correlation between receptor number and hypoxia survival times on Day 60 may be explained by the fact that following DFP treatment, the amount of acetylcholine in the CNS (which contributed to prolonged hypoxia survival times at the other time points) was gradually reduced as new molecules of acetylcholinesterase were synthesized.

This study has characterized the time course of development and recovery of central cholinergic subsensitivity to chronic DFP administration in mice. This cholinergic subsensitivity (reduction in the number of central muscarinic receptors) may be similar to that seen in Alzheimer or senile dementia patients (7-9) and thus this model may serve as a screen for pharmaceutical agents that could be developed to treat or prevent Alzheimer's disease. The DFP-induced cholinergic subsensitivity model has the added advantage of circumventing the expense of utilizing naturally aged animals for these studies.

The hypoxia screen was evaluated for its ability to reflect small changes in the number of muscarinic receptors in mouse cerebral cortex. This study suggests that the hypoxia survival times may be sensitive indicators of central cholinergic function, but are not a clear predictor of the level of muscarinic receptor binding in cortical homogenates. Further studies are needed to assess the utility of the hypoxia screen as an indicator of the number of central cholinergic receptors.

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