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ORGANOPHOSPHATE TOXICITY: GENETICS, RECEPTORS, AND ANTIDOTES

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Studies were completed on the time course of the effect of DFP on brain acetylcholinesterase ACH activity. While brain ACH activity is maximally depressed only 5 minutes after administration, most behavior effects require two or more hours before the maximal effect is seen. Following a 6.33 mg/kg dose of DFP the activity of brain ACH does not return to control levels, even after 30 days. This effect is dose-dependent and affects all brain regions equally. Behavioral studies as well as studies to demonstrate tolerance to DFP have been conducted. Pyridine Chemistry has been studied in an attempt

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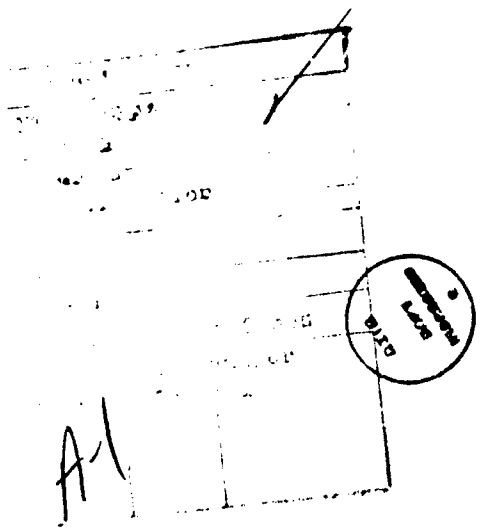
to develop nictonic receptor agonists.

PROGRESS REPORT

Organophosphate Toxicity: Genetics, Receptors, and Antidotes (AFOSR-82-0300)

September 15, 1982 - September 14, 1983



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INTRODUCTION

The following report presents a detailed analysis of the progress made in the first 14 months of our AFOSR-sponsored research. Our original proposal had four specific aims. These were:

1) Characterize nicotinic receptors in mouse brain.
2) Assess genetic influences on organophosphate toxicity.
3) Synthesize a series of agents that would inhibit nicotinic receptor binding.
4) Determine the efficacy of "binding inhibitors" as antidotes to organophosphates.

The first two of these specific aims are under the direction of Dr. Collins and the third is directed by Dr. Ruth. Specific aim 4 will be supervised by Dr. Collins but no compound has been synthesized yet that has proceeded this far in the testing. The major progress made has been on specific aims 2 and 3. Methods required to make progress in specific aim one are being developed and the methodology needed to assess antidotal effectiveness (specific aim 4) has been developed. In essence, we believe that we are off and running at a very good clip. The following report enumerates our progress in detail.

Time course of effect of DFP on brain acetylcholinesterase activity. It is well known that DFP inhibits both serum cholinesterase and acetylcholinesterase. Chronic treatment with DFP requires multiple injections of the drug. We wished to inject DFP in saline if possible. Injecting DFP subcutaneously in an oil vehicle is considerably more time consuming, messy and most importantly, stressful to the animals. Figure 1 shows the effect of intraperitoneal (i.p.) injections of DFP in saline on brain acetylcholinesterase activity in male DBA/2, C57BL/6 and C3H/2 mice given 5.28 mg/kg DFP by intraperitoneal injections in saline. Brain acetylcholinesterase activity was determined by Ellman's method 2, 5, 10, 20, 30 and 60 minutes and 24 hours after injection. Figure 1 shows that within 5 minutes of injection brain acetylcholinesterase activity was maximally inhibited, and that the activity remained at this level for approximately 24 hours. This shows that intraperitoneal injections of DFP prepared in saline are suitable for markedly reducing brain acetylcholinesterase activity quickly and continuously. It is interesting to note that while brain acetylcholinesterase activity is maximally depressed only 5 minutes after administration, most of the behavioral tests require two or more hours before the maximal effect is seen. (See later section).

Lethal effects of DFP. The LD_{50} for DFP was estimated in 3 inbred mouse strains: DBA/2, C57BL/6, and C3H. The DFP was prepared in saline at a concentration such that the animals were injected intraperitoneally with 0.01 ml of drug per gram of body weight. Six doses were used: 4.22, 5.28, 6.33, 7.38, 8.44, and 10.55 mg/kg (these doses are the result of making a simple 1:100 dilution of pure DFP in saline and diluting from there). Mice were kept 2 or 3 to a cage and observed for 3 hours. Time of injection and death were noted. Several animals died the next day, but these have not been included in the present analysis (these data are available if necessary). Twelve to 20 mice were used for each point.

Figure 2 shows the data plotted as dose of DFP vs % of animals dead at each dose of DFP. This plot is shown for clarity, although the actual LD_{50}s were calculated from linear regression analysis of log dose vs probit plots of the data. The DBA strain is the most resistant and the C57BL the most sensitive to DFP. This observation is borne out visually as well. The DBA mice at doses of DFP lower than 8.44 mg/kg were not markedly affected by the drug. They rested quietly in the cage and showed few external signs of anticholinesterase poisoning. The C57BL mice, however, were markedly affected with salivation, intense lacrimation (eyes white and opaque), severe diarrhea, extreme respiratory distress with obvious and copious respiratory tract secretions and severe tremors. The C3Hs showed signs intermediate between the two extremes. The time required for death to occur is shown next to the data point on Fig. 2. This shows that at any given dose, the DBA mice survived longer than the other two, a further demonstration of their relative resistance.

Female DBA and C3H mice are more resistant to the lethal effects of DFP than are the males. In the C57BL strain the opposite is true. This finding is of doubtful significance considering the steepness of the dose-response curve, which tends to overemphasize small changes.

The effect of DFP on body temperature. Figure 3 shows the hypothermic response of male and female DBA, C57BL, and C3H mice to a single intraperitoneal injection of DFP in saline. These experiments were conducted in a room constantly maintained at 23 ± 0.1°. Mice were weighed and placed back in their home cage. Two hours later their basal rectal temperatures were measured. Immediately after the basal temperature was taken, they were given DFP. Four doses of DFP were used: 3.17, 4.22, 5.28 and 6.33 mg/kg (on the figure these doses are referred to as their integer values only). Temperatures were taken at 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hours post injection. The smaller open and closed symbols represent saline-injected controls. Standard deviations are shown. Data were analyzed by 3-way analysis of variance.

Figure 3 shows little, if any, sex difference in the hypothermic response to DFP. There was no main effect of sex in the analysis of variance even though the females had consistently higher temperatures than the males at each time point.

Strain differences are clearly demonstrated. The C57BL mice were markedly affected by DFP as evidenced by the large temperature decrease. The C3H mice were very resistant to the hypothermic effects of DFP and the DBAs had a response intermediate between the two. This same rank ordering of these three strains has been observed before the nicotine induced hypothermia. As noted above, the C57BLs were visibly debilitated by DFP, the DBAs showed little outward signs of DFP intoxication and the C3H mice had intermediate signs (lacrimation and respiratory distress).

A dose-response relationship is also clearly demonstrated. Temperature decrement is greater with increasing DFP dose. In the case of the C57BLs temperature ranged down to 25° at the highest dose used. Even with such severe drug-induced effects, most of the animals survived, and by the next day their temperatures had returned to normal. At each dose the rank ordering of the strains remains with the C3H's being the least, and the C57BL's the most affected. During the course of this study and the preceding LD_{50} study, we measured the body temperature at the time of death in some of the animals (the temperature probe equilibrates within 5 sec). Interestingly, the temperature at the time of death is no lower than would be expected from the time-temperature curve of a living animal given a similar (or lesser) dose of DFP.

The time course for the effects of DFP on temperature shows that maximum response is attained after about 2 hours. In future studies in which tolerance to chronic DFP treatment will be assessed, temperature effects will be measured at two hours post DFP administration. These hypothermia profiles show a clear genetic influence on the response of inbred mouse strains to the acute, sublethal effects of DFP. We shall exploit these differences in the chronic treatment studies which are in progress.

Figure 4 shows the brain acetylcholinesterase activity remaining in the animals used in the temperature profiles. The next morning (temperature returned to normal) the mice were killed by cervical dislocation, the brain removed, homogenized in hypotonic phosphate buffer, and acetylcholinesterase activity measured by Ellman's method. It is clear that acetylcholinesterase activity decreases in a dose-dependent manner.

Figure 5 shows these data in a slightly different form. Body temperature 2 hours post DFP administration (maximum effect) is plotted against brain acetylcholinesterase activity 24 hours following DFP. All four doses are plotted for all three strains. Males and females have been plotted separately even though there is no statistical difference between them as determined by comparison of the slopes of the lines. This plot indicates that maximum temperature depression is linearly related to the degree of acetylcholinesterase inhibition and the effect is the same in each of the strains. ACHE activity was measured the next day. In each case the acetylcholinesterase activity is markedly depressed, but the body temperatures have returned to normal. This indicates that acute tolerance to the hypothermic effects to DFP develops in each of the strains and implies a modification of some acetylcholine receptor coupling mechanism occurs rapidly. In the future we shall attempt to ascertain the magnitude, time course, and mediator of this acute tolerance.

Time course of recovery of acetylcholinesterase activity following a single intraperitoneal injection of DFP.

In order to chronically treat mice with DFP we must inject sublethal doses at some given time interval. Most of the previous work was done with rats and doses of 1 or 2 mg/kg are given every other day. At these doses it is reported that rats show signs of acute organophosphate intoxication. We have found that doses of DFP less than 3 mg/kg cause almost no visible effects on mice, except for some sedation. We wanted to choose a dose which would lower acetylcholinesterase activity to 25% of control and select a dosing interval to keep the activity at that level. We wished to wait as long as possible between injections to minimize stress of treatment (we will discuss this in detail in a section on chronic DFP treatments).

To pick a dosing schedule logically we needed to know what the kinetics of return of enzyme activity were in the various mouse strains. We chose a dose of DFP which would cause a maximal decrease of brain acetycholinesterase activity without being fatal. We chose 6.33 mg/kg. Male mice were injected with this dose i.p. and brain enzyme activity was measured after 1, 2, 4, 6, 13, 20 and 30 days.

The data for all three strains are shown in Figure 6. We have represented these data on a linear axis since we assume that recovery of the enzyme activity should follow zero order kinetics. In fact, if the data are plotted on log-linear axes, the fit is quite poor.

Figure 6 shows that following a 6.33 mg/kg dose of DFP the activity of brain acetylcholinesteras does not return to control levels even after 30 days. The enzyme activity increases linearly for about 6 days, after which it plateaus at approximately 70% of the control value. For all practical purposes, then, the enzyme activity never returns to normal after a single exposure to DFP. We were quite surprised at this finding. A search of the literature revealed that others have also found this result (using several different organophosphates) both in CNS and peripheral tissue (e.g. diaphragm), but no one seems to have thought much about this. We, however, feel that this may have far reaching, even ominous consequences. Why has the enzyme activity not returned to normal? Could it be that DFP exposure results in the death

of a susceptible population of neurons and that the failure of acetylcholinesterase activity to return to control is the result of death of 30% of the cells which once had acetylcholinesterase activity? We think it an important question to ask, and it is one which we plan to investigate vigorously.

Figure 7 is a dose-response curve showing brain acetylcholinesterase activity 13 days following a single intraperitoneal injection of 3.18, 4.22, 5.28 or 6.33 mg/kg DFP to male DBA mice. A clear dose-response relationship is evident, and the enzyme activity appears to reach a limiting value of 4.0-4.5 μmoles/hr/mg protein. Higher doses of DFP are not feasible since too great a percentage of animals die at doses in excess of 6.33 mg/kg. We believe these data are consistent with the hypothesis that DFP is cytotoxic to a select group of neurons and that the cytotoxic effect is dose dependent.

Figure 8 shows the effect of a second injection of DFP. Male DBA mice were injected with 6.33 mg/kg DFP. On day 10 they were given a second injection of the same dose of DFP. Brain acetylcholinesterase activity was measured 1, 6, 13 and 20 days after the second injection (which is 11, 16, 23 and 30 days after the first injection). Figure 8 shows that following the second dose of DFP, the enzyme activity was markedly reduced (to approximately 10% of the control activity). The acetylcholinesterase activity increased linearly and eventually reached the same level of activity as the single-injected animals. This is further evidence that DFP destroys a select group of sensitive cells. If the DFP were acting as a general cytotoxic agent we would have expected that on the second injection the enzyme activity would eventually plateau at a level lower than that found after a single injection, which clearly did not occur.

All of the above results were obtained from whole brain. We were interested in knowing if the reduction of brain acetylcholinesterase activity occurred specifically in one of more regions, or was a general phenomenon affecting all brain regions equally. Male DBA mice were given DFP, 6.33 mg/kg, controls received saline. Thirteen days after the injections, the mice were killed and the brains were dissected into cortex, hindbrain (pons-medulla), hippocampus, hypothalamus, striatum and midbrain (those structures which remianed after removal of hippocampus, striatum and hypothalamus). Acetylcholinesterase activity was measured in each region and the results are reported in Table I. As can be seen, all brain regions are affected equally by DFP.

We have shown that the acetylcholinesterase activity in male mice does not return control after as long as 30 days following a single exposure to DFP. We have also shown that this effect is dose-dependent and affects all brain regions nearly equally. Figure 9 shows the rate of return of acetylcholinesterase activity in the brains of female mice following a single 6.33 mg/kg dose of DFP. As can be seen, the enzyme activity in the brains of the females returns to control within 30 days (compare to Figure 6). The conclusion follows that males and females are affected differently by DFP: that females are able to fully recover from a sublethal exposure to DFP while males remain impaired for a long period, perhaps forever.

If there is a sex difference in the response to a single exposure to DFP, this is an interesting and important finding. We are currently repeating this experiment with the females to be sure that they do in fact return to normal. In the very near future we will conduct an experiment to determine if castration of adult males changes their response to DFP. Male DBA mice 50-60 days of age will be castrated and after a five-day recovery period will be given a single intraperitoneal injection of DFP. For the control group we will use their littermates which will undergo sham operation and treatment with DFP. Saline controls will be run as well. This is the first experiment we will run in an attempt to determine the possible hormonal influences on this response to DFP.

Behavioral and Physiological Tests.

In order to determine whether chronic DFP treatment affords tolerance to that (or other cholinergic drugs), we use a multifactorial test battery which includes the measurement of body temperature and heart and respiratory rates following drug challenge. The effects of the drug on two motor tasks, the ability to walk on the rotarod and Y-maze activity, are assessed. Y-maze activity is composed of two measures: one a total activity measurement, and the other the total number of rears during the 3 minute test. We find these six measurements all have their particular advantages in assessing tolerance development. For example, rotarod score and heart rate are especially good measurements of tolerance to muscarinic agonists whereas respiratory rate and Y-maze activity are good for measuring tolerance to nicotinic agonists. Body temperature is an excellent measure for both.

In an effort to minimize time and cost, and maximize the amount of information gained per animal tested, we have examined the possibility of using a within-subject-repeated-measure experimental design for testing using the test battery. We were primarily concerned whether an animal could be administered the test battery on three consecutive days without carryover effects (tests X trial interaction).

In order to assess this possibility 70 male DBA mice were randomly assigned to one of the following seven groups:

	Day 1	Day 2	Day 3
(1)	saline	nicotine	oxotremorine
(2)	saline	oxotremorine	nicotine
(3)	nicotine	oxotremorine	saline
(4)	nicotine	saline	oxotremorine
(5)	oxotremorine	nicotine	saline
(6)	oxotremorine	saline	nicotine
(7)	saline	saline	saline

The first six groups present two cholinergic drugs and saline (we want each animal to act as its own saline control) in every possible order. This protocol allows us to determine the best order to administer the challenge drugs and to assess behavioral and physiological tolerance to nicotinic and muscarinic agonists. The seventh group, receiving saline once per day for three consecutive days, was included as a direct test of carryover effects from the repeated

measure design. Each mouse was tested on three consecutive days following a single i.p. injection of physiological saline, nicotine (2 mg/kg), or oxotremorine (0.2 mg/kg). All solutions were administered in a volume of 0.01 mg/kg. Thus the two drugs and saline were administered to the mice over the three day period such that each mouse received each substance once during the three day test period. Following injection with either drug or saline the mice were administered the test battery. Time-course studies with nicotine and oxotremorine resulted in the following test (and time) order for nicotine-treated mice: respiratory rate at 1-2 min, rotarod at 2.5-4 min, Y-maze activity and rearing at 4.5-7.5 min, heart rate at 8.5 min, and body temperature measurement at 15 min. Similarly, the following timing was determined for oxotremorine-treated mice: respiratory rate at 16-17 min, rotarod at 17.5-19 min, Y-maze at 19.5-22.5 min, heart rate at 23.5 min, and body temperature at 30 min. These times were chosen to coincide with the maximal effects of these drugs as measured by the test battery.

The results of this experiment are summarized in Fig. 10. These data are being analyzed using multivariate analysis of variance, however, one can see by visual inspection that an animal can be tested on three consecutive days without significant carryover effects. Only in the case of rotarod performance does there appear to be an improvement over trials. Thus, in an attempt to avoid confounding learning with drug effect, in subsequent tolerance studies nicotine challenge will always occur on day 1 of testing, following by saline on day 2, and oxotremorine on day 3.

Time-course of DFP effects. Our initial studies have been concerned with the characterization of the response of mice to DFP using the multiple variable test battery described in the methods section of our proposal. This behavioral and physiological test battery was developed to ascertain the relative contributions of nicotinic and muscarinic actions to DFP response and includes six measures: respiratory rate, heart rate, body temperature, rotarod performance, Y-maze activity, and rearing in the Y-maze. In order to determine the optimal post injection testing times, time-course studies were conducted following a single, acute i.p. injection of DFP. Three doses of DFP were used in these time course and dose response studies: 3.0, 4.0 and 5.0 mg/kg. Most of this study was done with male DBA mice. The data are summarized in Figures 11 and 12. Each point is the mean of 7-10 mice per time for the DFP-treated groups and 3-7 mice per time point for saline-injected controls. Each animal was examined on one of the six tests included in the battery. In most cases individual mice were monitored throughout the entire time course. We have found this to be particularly important for Y-maze activity and rearing in the Y-maze, both of which are significantly affected by repeated testing over a six hour time period. In general, repeated testing has little effect on the remaining four tests. It is of interest to note that Y-maze activity and rearing are not affected by repeated testing if mice are tested once per day on three consecutive days (Fig. 10 b & c).

The results suggest that respiratory rate, Y-maze activity, rearing in the Y-maze, and rotarod performance are rapidly decreased following acute DFP administration. Respiratory rate falls from a basal rate of 250 to 140 breaths per minute within 12 min post injection at the 4 mg/kg dose (Fig. 11). Respiration remains depressed for approximately two hours. It slowly returns to normal levels in approximately six hours.

A steady decline of heart rate is observed during the first hour post DFP injection (Fig. 10). We have measured heart rate over a seven hour time period and have found it to be depressed, relative to saline-treated controls, throughout the test period. As can be seen from Fig. 10 it appears that the heart rate is slowly returning to pre-DFP levels, however, our most recent data suggest that the heart rates of some DFP-treated DBA mice have not returned to normal levels 24 hours following DFP exposure. Although we do not have enough 24 hr heart rate data to warrant inclusion in this progress report this is an interesting finding which deserves further study.

Both Y-maze activity and rearing within the Y-maze are maximally depressed by 15 min post DFP injection (Fig. 12) and remains so for approximately one hour following which modest increases are observed. Locomotor activity in the Y-maze for both DFP and saline-treated mice coincide by five hours post treatment. As previously stated this represents a modest increase in activity for the DFP-treated mice but a large decrease in the activity of the saline controls, reflecting the effect of repeated testing in the Y-maze over a short period of time (6 hrs.). A similar trend is observed for rearing in the Y-maze. It should be noted that following the 3 mg/kg and 4 mg/kg doses of DFP, the DBA mice in the Y-maze do not appear debilitated and are able to move with speed and ease during attempted transfers into and out of the apparatus. Although they are physically capable of movement, the DBA mice lose their ability to walk on a rotating rod. The impairment of rotarod performance is maximal 15-30 min post DFP administration but returns to pre-treatment criterion level (100 sec) by four hours post DFP injection for the 3 and 4 mg/kg doses. For the 5 mg/kg dose the animals are still not to criterion by four hours, but are steadily improving. The effects of DFP on body temperature have been discussed in a previous section.

All of the physical and behavioral parameters which we have looked at are maximally reduced by DFP by 2 hours. To assess tolerance development to the effects of DFP, we will challenge the mice with DFP and measure their responses starting 2 hours post injection. A dose of 4 mg/kg will be used. The 3 mg/kg dose does not depress the responses enough to be useful, and the 5 mg/kg dose depresses them too much to be useful.

Chronic treatment of mice with DFP. Since DBA mice show marked development of tolerance to nicotine and oxotremorine, we initiated our chronic treatment studies with them. Male DBA mice were treated chronically with DFP using intraperitoneal injections of the drug in saline according to the following schedule: 2 mg/kg on day 1, 1 mg/kg on days 2 and 3, and 2 mg/kg on days 5, 7, 9, 11 and 13. Saline controls were run concomitantly. On day 14 the animals were given a challenge dose of nicotine (2 mg/kg) and run through the behavioral test battery (respiratory rate at 1-2 min, rotarod at 2.5-4 min, Y-maze activity at 4.5-7.5 min, heart rate at 8.5 min, and temperature at 15 min). That afternoon mice were injected with DFP (1 mg/kg) or saline (controls). On day 15 animals were injected with saline and run through the test battery using the same times as for the nicotine challenge. A 1 mg/kg injection of DFP (or saline) was administered in the afternoon. On day 16, mice were challenged with oxotremorine (0.2 mg/kg). Tests were performed 15 min after injection using the same time intervals as for nicotine and saline (e.g., respiratory rate at 16-17 min, temperature at 30 min). Results are tabulated in Table I.

The DFP-treated animals are somewhat less affected by nicotine than are the controls. This reaches significance only in the respiratory rate measurement in which the DFP-treated mice do not show nicotine-induced stimulation of respiration. Heart rate is not reduced as much in the DFP group, but this does not reach statistical significance ($P = 0.057$). Body temperature is not reduced as much with the treated mice althqugh this too is not statistically significant. Rotarod scores are lower for the DFP-treated mice. This is due in part to the propensity of the DFP animals to jump (not fall) off.

Saline baselines show little difference between saline controls and DFP-treated groups. It should be noted that the DFP-treated animals are not debilitated (they are practically impossible to catch, for example). The significantly reduced Y-maze scores of the DFP group below control is due simply to the unwillingness of the treated mice to move when unprovoked.

It should be noted that in addition to treating mice with DFP in saline, we also ran two groups (total of 12 mice) which were injected with DFP delivered subcutaneously in a corn oil vehicle. We are concerned that route of administration might be important. These animals were indistinguishable from the saline-vehicle groups except that the oil-injected mice were considerably more difficult to handle. A potential problem in these treatments is the number of times the mice are handled and injected. As a rule, intraperitoneal injections of mice are not particularly stressful to them. We did not even pick them up to inject (just lift the tail and inject into the abdomen--they don't even turn around). After the 6th or 7th injection, however, the mice become quite agitated, and even the saline controls become difficult to manage. This difficulty in handling results in more stress to the animal. Their behavior becomes quite abnormal which affects their test scores. This problem is clearly deomonstrated in the next section in which we describe our attempts to show behavioral tolerance to DFP challenge in animals chronically treated with DFP according to the schedule describe above.

DFP tolerance. Following 14 days of chronic DFP treatment, tolerance to DFP was assessed using the multivariable test battery previously described. On the first day of testing the mice were weighed and injected with physiological saline. The test battery was administered at the following times: Y-maze activity, 30 min; rotarod performance, 33.5 min; respiratory rate, 120 min; body temperature, 123 min; and heart rate, 125 min. After completing the test battery, the mice were returned to their home cages until the second day of testing . On the second day of testing the mice were weighed, injected with 4 mg/kg DFP (0.01 mg/g body wt), and the test battery was administered a second time. The results of this experiment are summarized in Fig. 13.

The histograms represent the mean $\pm$ SEM for each test. The black bars represent mice chronically treated with DFP while the open bars represent the saline-treated controls. To reiterate, on day 1 both the DFP and saline-treated mice were injected with saline and on day 2, both the DFP and saline-treated mice were administered a challenge dose of DFP. We anticipated that on day 1, the mice which had been chronically treated with DFP would have lower scores on all of the tests than the mice treated chronically with saline and on day 2 the tolerant DFP mice would out-perform the saline controls which were experiencing DFP for the first time. The results, however, were quite surprising. As can be seen from Fig. 13, there were no differences between

DFP and saline controls on either day for five out of the six tests. Body temperature (Fig. 13e) was the only measure that differentiated the chronic DFP from saline control groups. Mice treated chronically with DFP exhibited higher body temperatures following both saline challenge (day 1) and DFP challenge (day 2) than did mice treated chronically with saline and challenged similarly. Within and DFP and saline groups there were no significant differences between day 1 and day 2. We were particularly surprised that the saline controls challenged with DFP (open bar, day 2) did not respond with a subsequent, dramatic drop in test battery performance. As can be seen from Fig. 13 mice chronically treated with saline and challenged with DFP (day 2, open bars) performed significantly better on all tasks compared to naive mice treated with an acute dose of DFP (dashed lines within the open bars). We believe this somewhat perplexing result to be an artifact of the treatment paradigm. As previously described, the mice undergo a 14 day injection regiment which has proven to be highly stressful to both DFP-treated and saline-injected controls. The stress of continued handling has clearly affected their test scores.

We were somewhat surprised at the small magnitude in the response to DFP. It could be that the DBA mice which are rather resistant to DFP do not develop much behavioral tolerance in response to chronic acetylcholinesterase inhibition. If this is the case, we would expect a strain which is quite sensitive to acute DFP exposure to develop more tolerance, and the C57BL strain might be a better choice. Alternatively, it could be that some of the effects are handling-induced. We decided to test both of these hypotheses in the next sets of experiments.

In order to reduce possible handling stress we administered a high dose of DFP using a longer dosing interval. Referring to Figure 6, we can see that doses of DFP of 4, 5 or 6 mg/kg reduce brain acetylcholinesterase activity to approximately the same level. Since 4 mg/kg is a dose which all of the strains handle easily, it was chosen as our standard treatment dose. The additional reduction of acetylcholinesterase activity with 5 mg/kg DFP is not worth the additional debilitation (which is considerable) it causes the animals. The dosing interval chosen was 5 days which was chosen from Figure 6. We are using the dosing interval with DBA, C57BL and C3H mice. These experiments are in progress now, and not all of the data have been collected or analyzed. It will take several more weeks to complete these studies, however much of the behavioral data has been collected and we shall briefly summarize these results.

Male C57BL, C3H and DBA mice were assigned to 2 different groups: one which would receive DFP and the other which would receive only the saline vehicle (control). Wherever possible we have chosen 2 littermates from each family and assigned one to the DFP and one to the control group. In this way we hoped to avoid any experimental bias resulting from possible within-litter effects. On day zero, animals were weighed and were injected intraperitoneally with either 4 mg/kg DFP (made up such that the animals were injected with a volume of 0.01 ml of drug solution per gram of body weight) or saline vehicle (again 0.01 ml per gram of body weight). This procedure was repeated on days 5, 10, 15, 20, 25 and 30 for a total of seven injections.

After the last injection, the mice were tested for tolerance to nicotine, oxotremorine and DFP using the test battery previously described. The DFP treated mice were divided into two groups. Group I received nicotine challenge on day 31, saline on day 32 and DFP on day 33. Group II received oxotremorine challenge on day 31, saline on day 32 and DFP on day 33. Also, the controls were divided into two groups and tested just as the DFP-treated animals were tested. Using this protocal half the DFP-treated animals were tested for tolerance to a nicotinic agonist, and half for tolerance to a muscarinic agonist. All were tested for tolerance to DFP, and all had a saline-baseline included. Likewise, their vehicle-treated, littermate controls were tested for tolerance to nicotine, oxotremorine and DFP. In this way we should be able to determine if chronic treatment with DFP conferred any tolerance to any of these drugs. The neurochemical studies are in progress and will take several weeks to complete.

The table which follows (Table III) includes all of these battery data for male DBA mice treated with DFP or vehicle and challenged with nicotine, oxotremorine and DFP. Saline baselines are included. It does not appear that DFP treatment conferred any degree of tolerance to nicotine, oxotremorine or DFP. We have tried 2 different approaches now for administering DFP, One a low dose, short interval procedure (Table II) the second a high dose, long interval treatment (Table III). With neither of these approaches have we been able to demonstrate significant tolerance development to DFP. We are using the high dose, long interval treatment schedule on male C57BL and C3H mice. These data are being processed now, but again we are finding no (or very little) development of tolerance to any of the these cholinergic drugs. The test battery we are using has been successful in the past to measure tolerance acquisition to a variety of nicotinic and muscarinic agonists as well as alcohol. These tests cover a wide variety of physiological and behavioral responses. If tolerance to DFP develops we should be able to measure it, and we have not been able to do so. After several treatments with DFP, the mice seem to be somewhat less impaired than they were at first, this may be construed as tolerance but the effect is not striking.

In the past, others have claimed to have shown development of tolerance to DFP, and we were troubled that we do not see it in our studies. A more critical examination of some previously published studies casts some doubt on the degree of tolerance developed in the hands of other investigators. For example, Costa et al (Toxicol. Appl. Pharmacol. 60: 441-450, 1981) treated mice with disulfoton and measured tolerance by resumption of weight gain and a shift in the LD80 for carbachol administered by i.p. injection. These are both reasonable things to measure, but are not all that convincing. Weight gain is a measure of recovery, not necessarily tolerance development, and carbachol does not pass the blood-brain barrier, so all its toxic effects are peripheral. It would have been interesting to see if the LD50 (or 80) for disulfoton was increased in their "tolerant" mice. We suspect it was not. Some of the most compelling evidence that tolerance to DFP develops is the often quoted work of Overstreet and his group (e.g. Psychopharmacology 65:15-20, 1979) who have selectively bred for sensitivity to DFP in rats. These investigators have developed 2 lines of rats termed "sensitive" and "resistant" referring to their responses to DFP. From the nomenclature, one would suspect that these investigators were successful in breeding for resistance to the initial effects of DFP. However, quoting from this paper (p. 15):

"Regression analysis confirmed that the selective breeding procedure was successful in establishing a line with increased sensitivity to DFP, but that it failed to produce a line more resistant than the original population."

And on page 19:

"Although the terms 'sensitive' and 'resistant' lines have been used throughout the foregoing paragraphs, the data suggest that only the former term is appropriate."

It is clear that the resistant line is only its name, and that it is just as sensitive to the toxic effects of DFP as the foundation population. This group has continued to use the terms "sensitive" and "resistant" in describing these lines of rats which we feel is, at best, grossly misleading. It is our opinion that tolerance to organophosphates has not been adequately measured by most groups which have attempted to do so. This is not to suggest that there is anything wrong with their data, however. We believe that the data itself is fine, but that the explanation for their findings is in error.

If, as we believe, that a single exposure to DFP destroys a select population of neurons, then many, if not all of the previous "tolerance" data can be explained by a reduction in the number of cells able to respond to the challenge drug. For example, is the shift in the carbachol LD80 curve due to acquistion of tolerance or due to a reduction in the number of responding cells? If the latter is true, does this truly qualify as tolerance? We think not. Our efforts in the upcoming months will focus on this question. Specifically, we will test the hypothesis that a single exposure to DFP can result in the same neurochemical adjustments as does chronic treatment.

Our approach will be as follows. If a single exposure to DFP destroys neurons, then the receptors associated with those cells should also be gone. Thus a reduction of 30% of the cells might result in a 30% reduction of receptor number which could be construed as down-regulation. Acute studies by others have measured cholinergic receptors 4 to 24 hours following DFP, and have shown no reduction in their numbers. What if we wait 7-14 days following a single dose? Would there then be a reduction in number? It is our prediction that there will be, and that "down-regulation" can occur after one exposure to an organophosphate. We will approach this subject from several directions. We will measure the activity of choline acetyltransferase and high-affinity choline uptake 13 days following a single dose of DFP. If presynaptic neurons are destroyed these should be the best measurements. If the post-synaptic neurons are destroyed it is a more difficult problem to investigate, but we should be able to provide enough indirect evidence to support the conclusion. At present we are establishing collaborations with investigators at other institutions to look at the brains of our treated mice histologically. We hope that it may be possible to demonstrate a reduction in neuronal density following a single high dose exposure to DFP. We anticipate sending sections of brain to an histologist to study. The histologist would be blind to the treatment group each specimen was in. We would try to visualize acetylcholinesterase and choline acetyltransferase and any other likely candidate to try to test our hypothesis. Additionally, we are working with Dr. Jeanne Wehner (IBG) to investigate the possibility of using

a tissue culture system to study this problem. Dr. Wehner's system is a rather unique one. Fetal brains are removed (day 17), the cells enzymatically dispersed, and allowed to reaggregate in culture. The result is a mixed brain cell structure approximately 1-3 mm in diameter which establishes neuronal connections and spontaneous electrical activity. All neurotransmitter receptors and associated enzymes are also present in these brain reaggregates. We are currently working on time courses and dose response curves for DFP in this system, and the work is progressing quite well. We are confident that the use of this cell culture system will be a valuable adjunct to our present whole animals studies.

We are well aware that we are progressing on a series of studies which were not outlined in our original grant proposal, but we feel that these are necessary and important studies to determine the effects of organophosphates on the CNS. In the next six months we shall:

1) Complete the chronic treatment studies with DFP in DBA, C57 and C3H mice, including the neurochemical measurements.

2) Study the return of acetylcholinesterase activity following a single dose of DFP. This will include a study on the effect of castration on the return of the enzyme activity.

3) Measure cholinergic receptors in the brain 13 days after a single injection of DFP. This will include some time-course and dose-response studies.

4) Measure choline acetyltransferase and high affinity choline uptake in brains of single-exposed animals.

5) Continue our efforts to demonstrate tolerance to DFP.

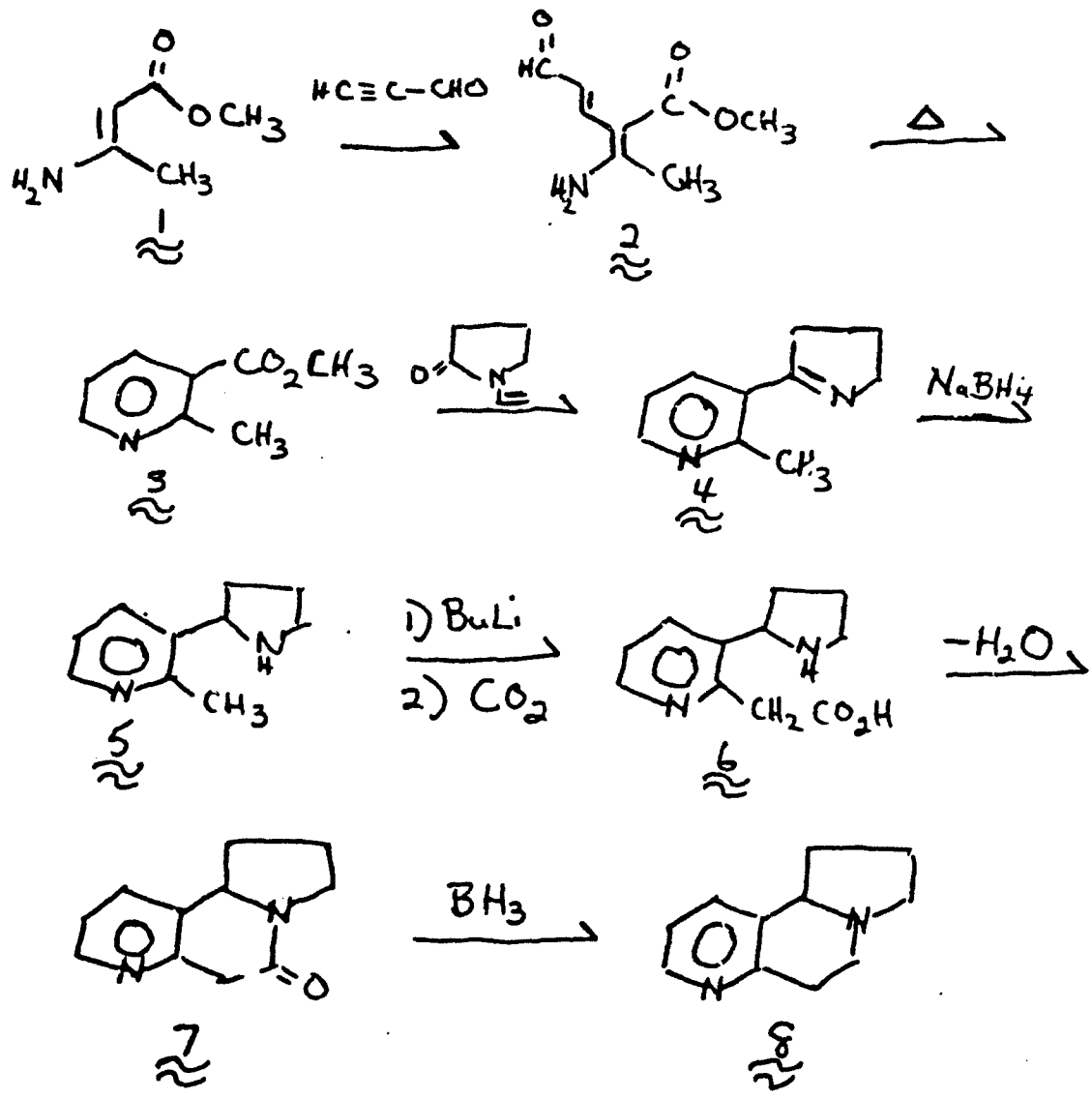
Since we believe that a single exposure to DFP results in some cell death, we must be quite sure that this observation is correct. We have repeated these experiments and will probably do so once again. Likewise, if males and females are different in this regard, we must be sure and another replicate is probably necessary. We have found little tolerance development to DFP. Since this will obviously be an unpopular result, we shall try to replicate tolerance tests used by others to see if we can get the same results. We shall most likely try the carbachol test of Costa et al, mentioned earlier. If we can find the same result, but offer a different explanation, our case will be strengthened.

In our original grant application we were concerned that there are no truly adequate antidotes for organophosphate poisoning. If it is true that organophosphates cause actual neuronal destruction, the search for an effective antidote is perhaps more urgent. The importance of this work pervades all of our lives every day. The possibility that our no-pest strips, garden sprays and flea collars are exposing us to low doses of organophosphates with potentially irreversible effects is a truly frightening prospect and one which requires thorough investigation.

CHEMISTRY SUB PROJECT

A number of aspects of pyridine chemistry have been explored in the process of developing routes to the target components listed in the proposal. In the process of developing these routes it has been necessary to explore and define areas of pyridine chemistry not existing in the literature. The following summary highlights these avenues of exploration.

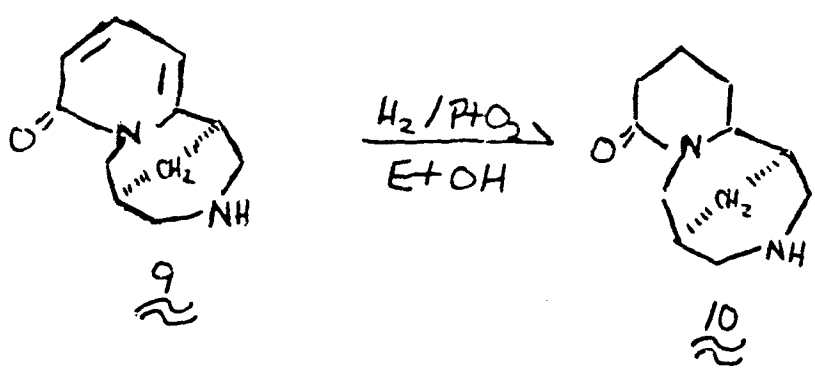
Scheme I



The tricyclic nicotine analog was prepared as illustrated in Scheme I. β-amino methylcrotonate (1) was condensed with propiolal to afford the 2-methyl nicotinate ester 3. This ester was converted to the pyrroline analog 4 by treatment with N-vinylpyrrolidone, which was reduced to the 2-methyl nornicotine 5 with sodium borohydride. The nicotine analog 5 homologated to the carboxylic acid 6 by treatment with n-butyllithium and CO_2. Dehydration afforded the tricyclic amide, which afforded the desired analog 8 upon reduction with borane.

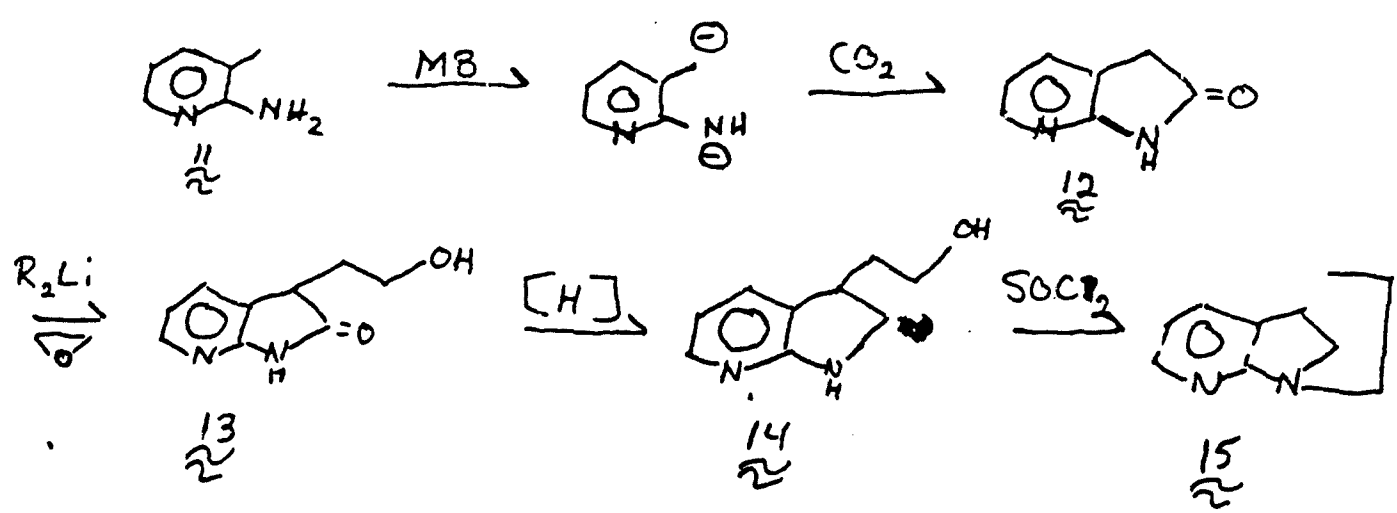
The tricyclic analog 8 has undergone preliminary study as a receptor ligand. The intermediate 4, 5, 6 and 7 will be examined in the near furture.

Scheme II



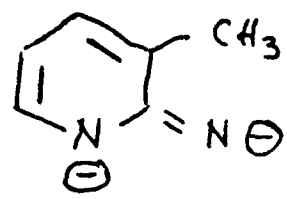
Catalytic reduction of the naturally-occurring alkaloid cytisine 9 to the tetrahydro deriviative 10 was unsuccessful (Sheme II) under atmospheric pressure. Higher pressure hydrogenation will be tried in the future.

Scheme III



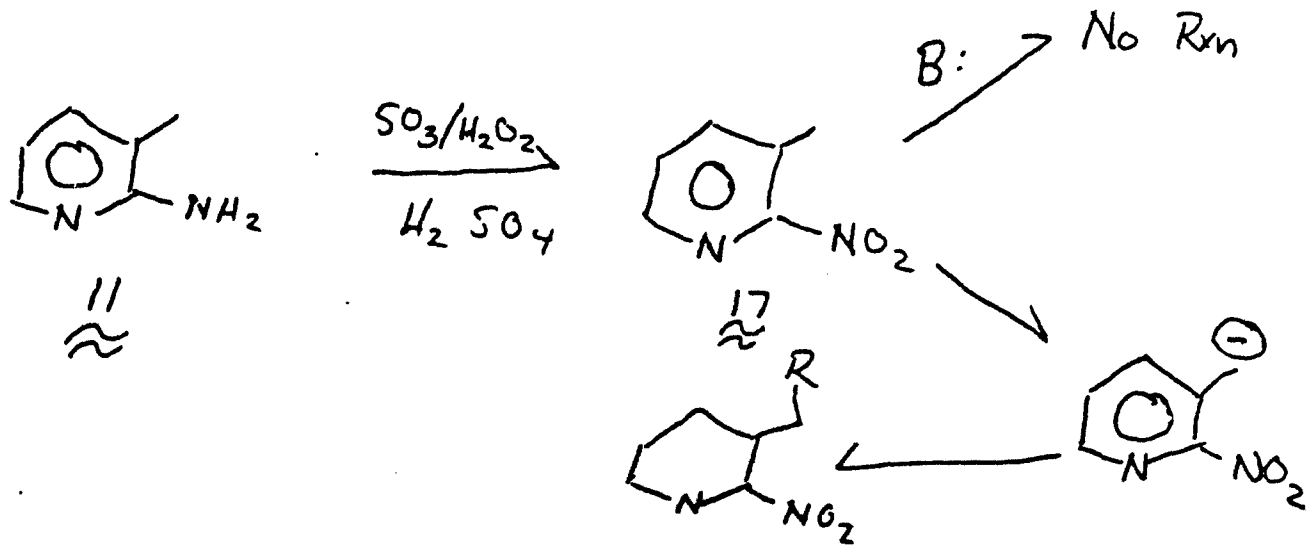
The synthesis of the bridged tricyclic analog 15 is nearly complete, but has required the exploration of several different possible routes. The initial approach is outlined in Scheme III.

The functionalization of 2-amino-3-picoline 11 to the lactam 12 via a dianion proved to be impossible due to 1) the lack of acidity of the 3-methyl hydrogens and 2) the apparent formation of the dianion 16.



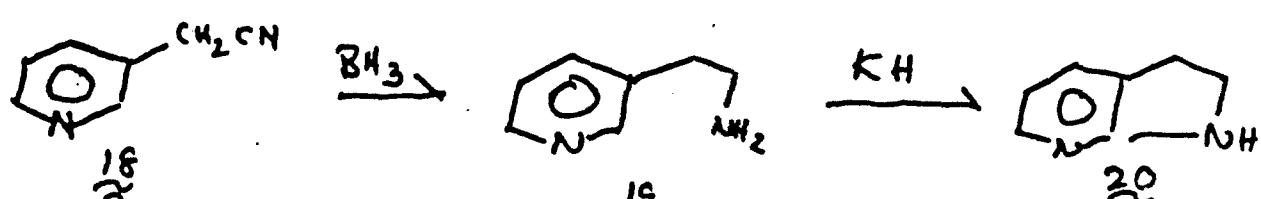
16

Numerous attempts to functionalize the 3-methyl group in 2-nitro-3-picoline 17 were unsuccessful, resulting in recovery of starting material.



11 17

Attention was then focused to the pyridineacetonitrile 18, and the possibility of conducting an intramolecular Chichibabin reaction, as shown in Scheme IV. Pyridineacetonitrile was reduced to the corresponding ethylamine derivative 19 in 80% yield with BH_3 Me_2S. Although yields were low, 19 was cyclized with n-BuLi to the dihydroazaindole 20. This reaction will be optimized since there are no



18 19 20

Scheme IV

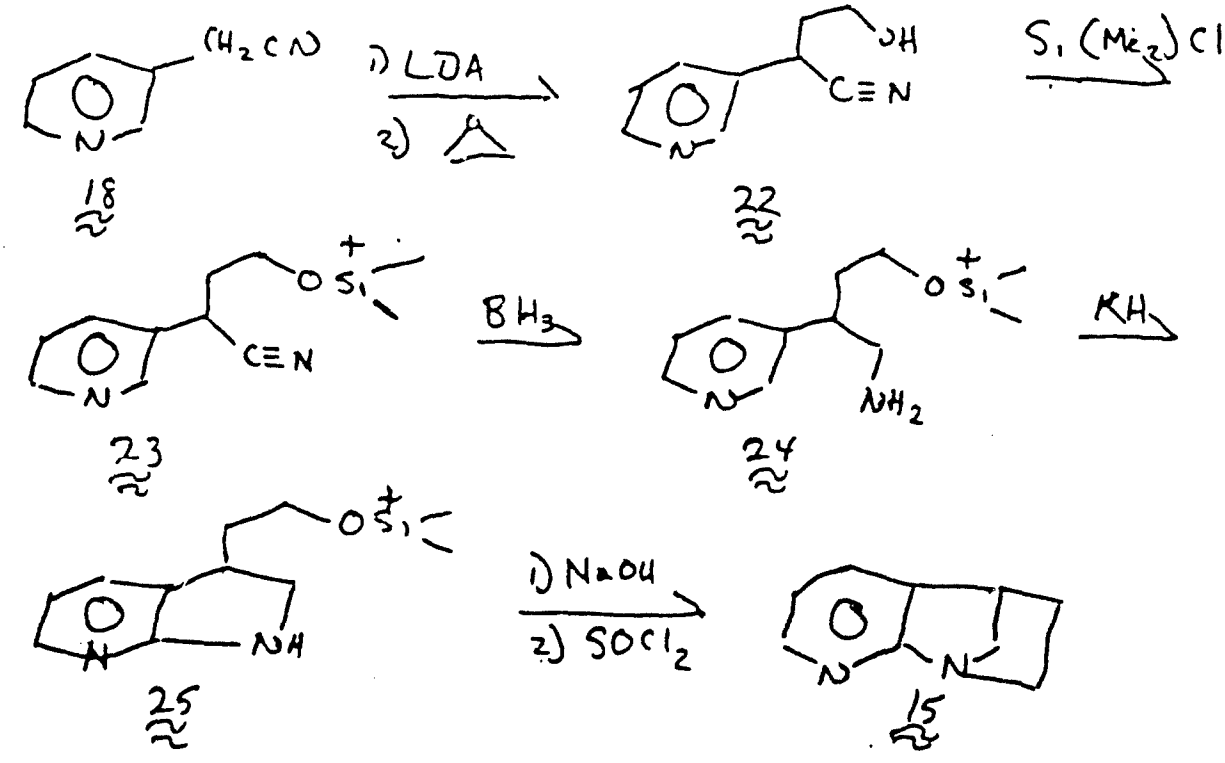
reports to date of intramolecular Chichibabin reactions. The structure of 20 was confirmed by synthesis from two other routes (Scheme V).

Scheme V

It was found that 20 could be generated by reduction of 7-Azaindole 21 with diborane. The only literature synthesis of dihydroazaindole 20 from 21 involved catalytic hydrogenation at 200 atm. Thus the borane reduction is of some practical importance. The dihydroazaindole 20 was also generated by borane reduction of the bicyclic lactam 12. The synthesis ofwhich will be discussed shortly.

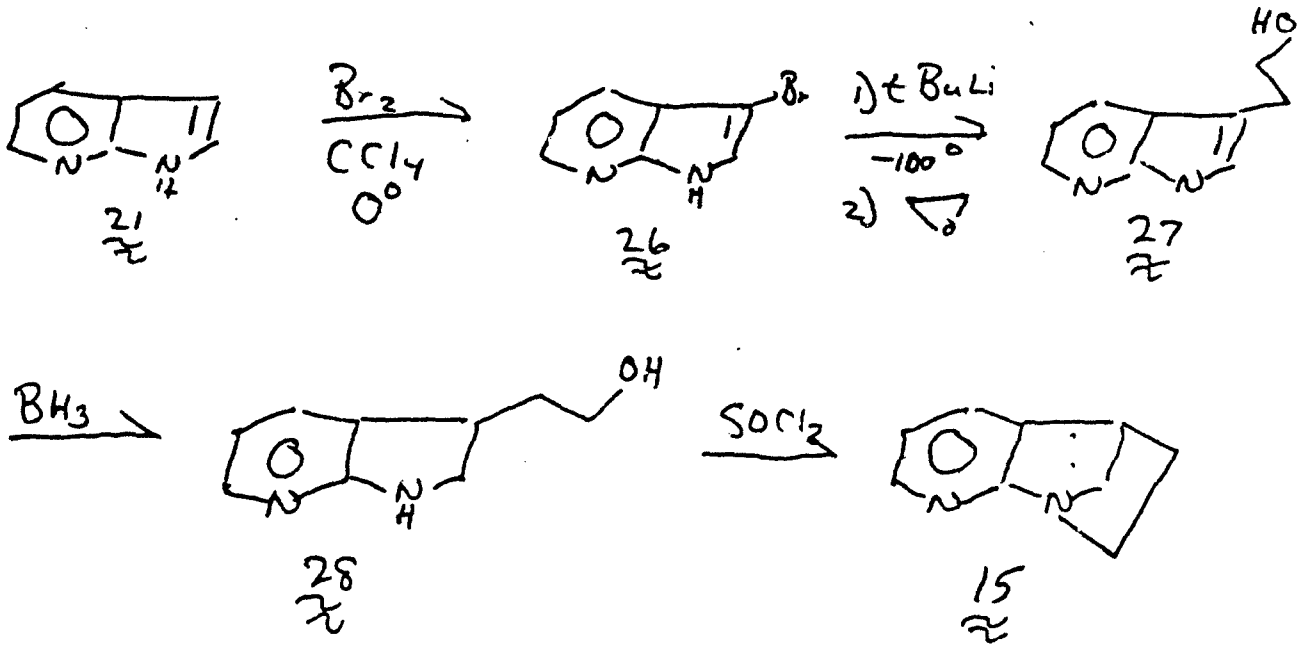
Knowing that the pyridly acetonitrile (18) could be reduced to the ethylamine with diborane, it was felt that the sequence outlined in Scheme VI could be employed to generate the denied analog 15.

Scheme VI



To this end, pyridylacetonitrile was clearly alkylated with ethylene oxide to the hydroxyethylnitrile 22 in 60% yield. The hydroxy nitrile was protected as a t-butyldimethylsilyl ether 23. However metal hydride reduction of the nitrile suprisingly promoted deprolection of the alcohol, and the formation of a number of side products. This route was temporarily set aside at the stage of the protected hydroxyethyl nitrile derivative 23, in hope that the sequence outlined in Scheme VII would be more fruitful.

Scheme VII

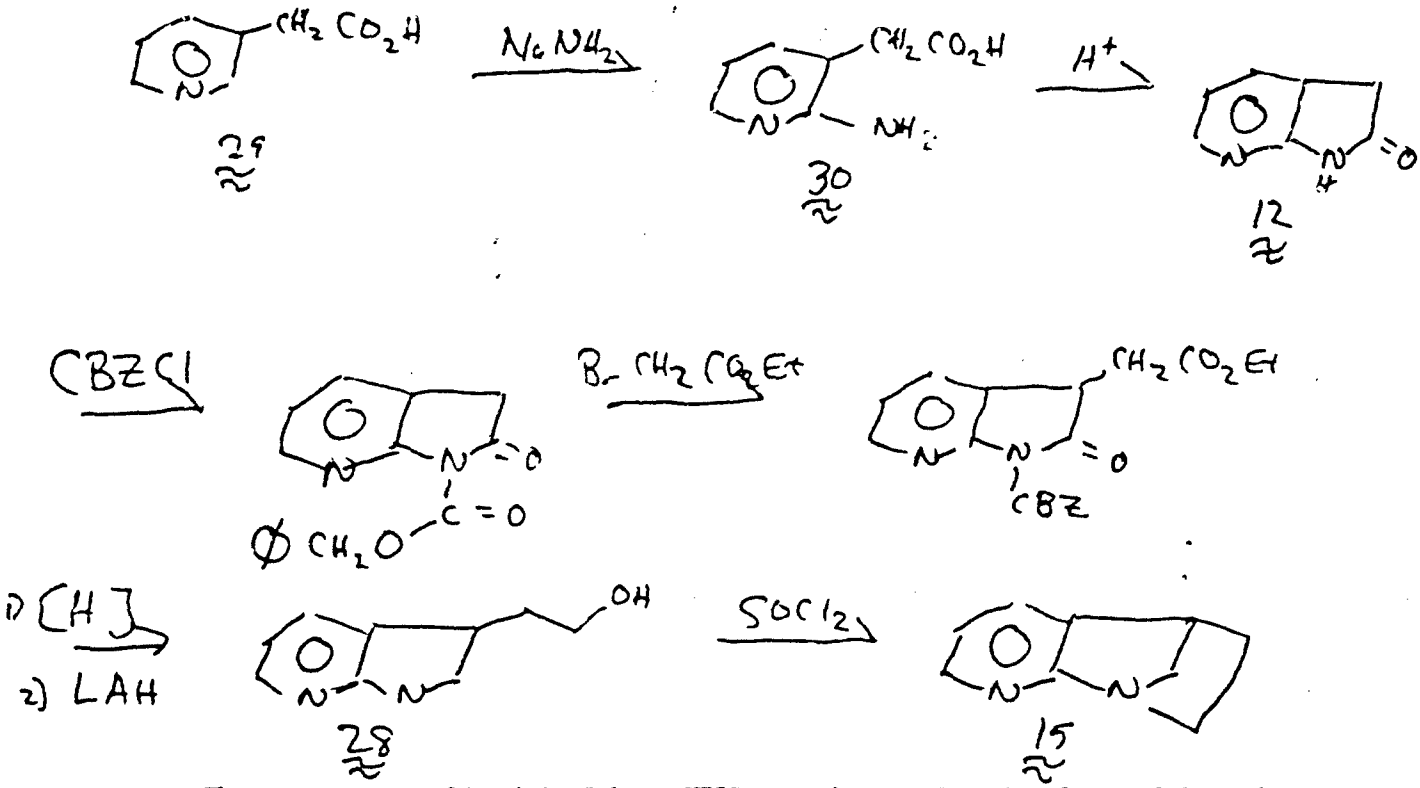


7-Azaindole (21) was cleanly brominated in carbon tet to afford the 3-bromo derivative 26. Metallation with t-Butyllithium at - 100°, followed by quench with ethylene oxide afforded the 3-hydroxethyl-7-azaindole 27 in good yield. Substitution of the 3-position, however, renders 2 unsusceptable to borane reduction under a variety of conditions.

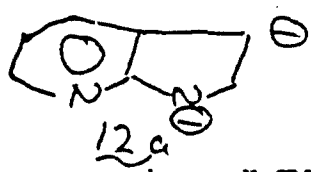
We have alkylated the borane intermediate from reduction of 7-azaindole

which has resulted in a complex mixture yet to be unraveled.

Scheme VIII



The sequence outlined in Scheme VIII was then explored. 3-pyridylacetic acid (29) was subjected to Chichibabin conditions to afford in moderate yield the 2-amino derivative 30. This amino acid could be cyclized to the corresponding lactam 12. Generation of a dianion from 12 (12a) has not been successful.

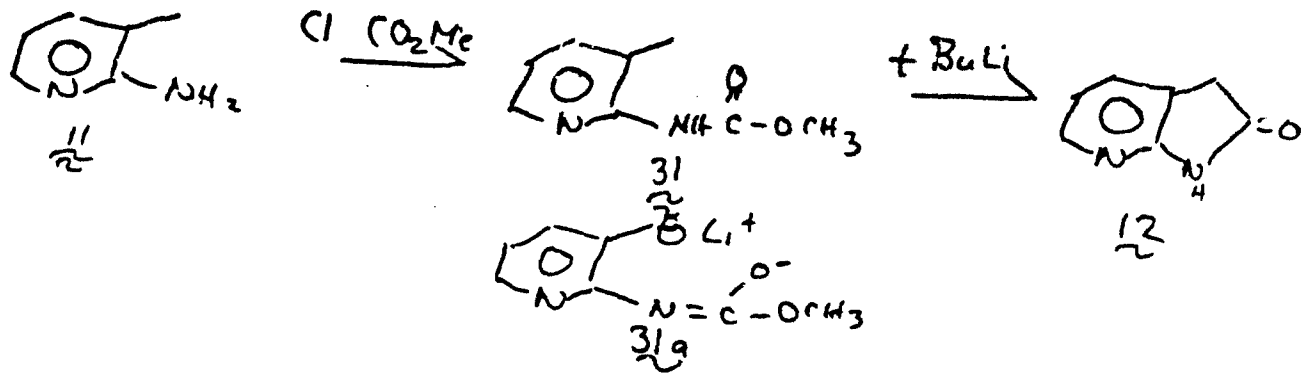


12a

Thus the lactam has been protected as a N-CBZ derivative which should allow ready alkylation of the 3 position. This route is currently the procedure being used to complete the desired analog 15. Completion is expected in the next month.

An improved method for generation of the lactam 12 has been developed as shown in Scheme IX.

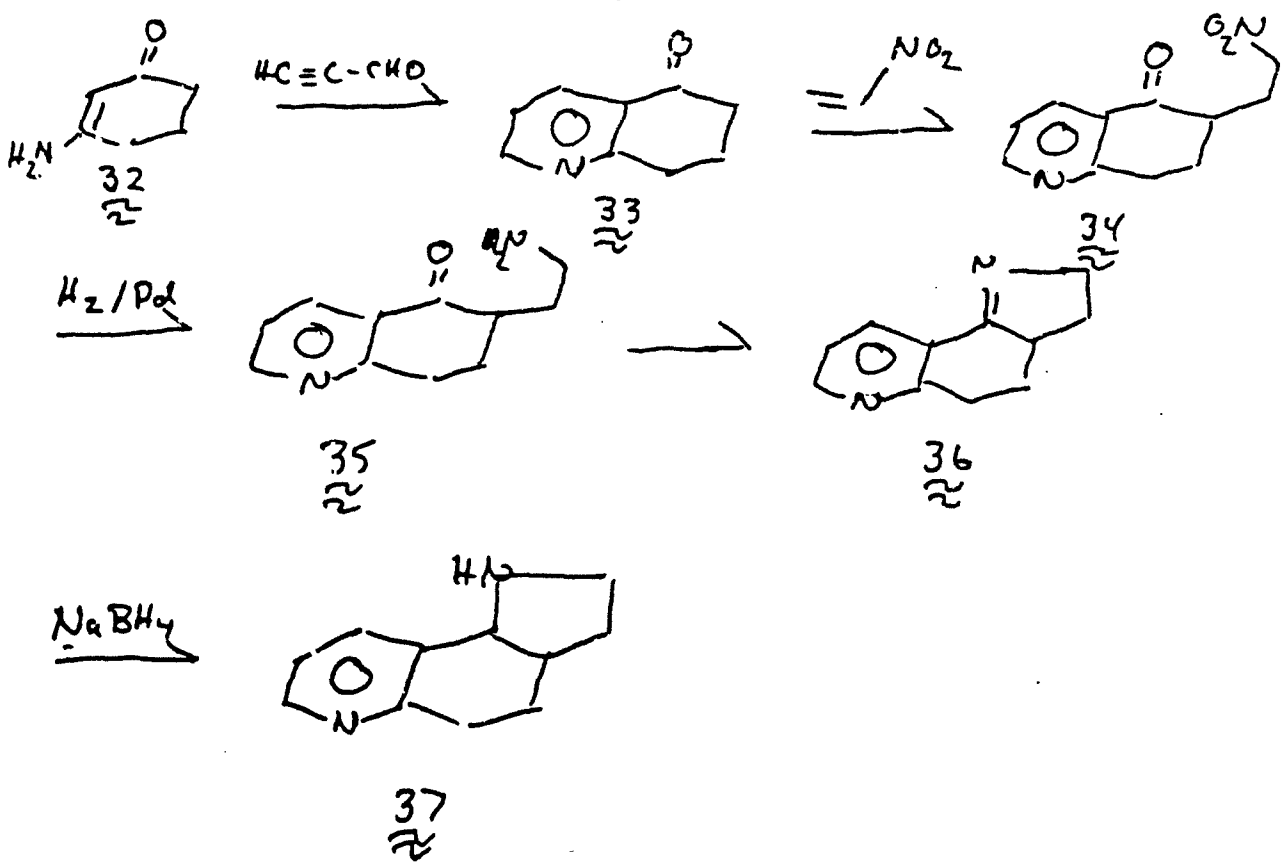
Scheme IX



2-Amino-3-picoline (11) was converted to the carbamate 31. While treatment of the carbamate with n-butyllithium resulted in addition of n-butyllithium to the carbonyl, treatment with t-butyllithium resulted in formation of the amide-directed lithiation product, the dianion 31a which underwent intramolecular cylization to 12 upon warming to room temperature. While yield are only modest, the route is direct, clean and inexpensive. Unreacted starting material is recovered and recycled.

A third analog is nearing completion as outlined in Scheme X.

Scheme X



The enamine of cyclohexinedione (32) was condensed with propiolal to afford the azatetralone 33. Condensation with nitroethylene, followed by catalytic reduction affords the aminoethyl homolog 35, which is condensed to the triaylic unine 36. Reduction then gives the nicotine analog 37.

TABLE I

Acetylcholinesterase activity in brain regions following DFP Administration

Region	Acetylcholinesterase Activity (μmol/hr/mg protein)		Acetylcholinesterase Activity
	Control	DFP	% Control
Cortex	7.52 ± 0.35 (8)	4.75 ± 0.23 (12)	63.2
Midbrain	7.94 ± 0.47 (6)	3.97 ± 0.05 (6)	50.0
Hindbrain	5.63 ± 0.15 (6)	4.49 ± 0.16 (6)	79.8
Hippocampus	4.52 ± 0.23 (8)	3.18 ± 0.28 (12)	70.4
Hypothalamus	5.31 ± 0.28 (6)	3.67 ± 0.10 (6)	69.1
Striatum	15.58 ± 0.70 (8)	10.86 ± 1.19 (12)	69.7

*Tabled values are mean ± SEM for the number of determinations given in parentheses. All assays were performed 13 days after a single i.p. injection of 6.33 mg/kg DFP or saline.

TABLE II

Measurements of Tolerance to DFP using Low Dose DFP Treatment

Challenge Drug	Group	Respiratory Rate (min^{-1})	Rotarod (sec)	Y-maze Activity	Y-maze Rearings	Heart Rate (bpm)	Temperature (°C)
Nicotine	Control	320 ± 16	78 ± 10	0.3 ± 0.3	0	545 ± 38	34.8 ± 0.5
	DFP	276 ± 10*	54 ± 9	2 ± 1	0.4 ± 0.2	616 ± 15	35.6 ± 0.3
Saline	Control	298 ± 12	87 ± 9	53 ± 7	26 ± 2	768 ± 13	38.5 ± 0.2
	DFP	300 ± 8	83 ± 9	37 ± 4*	14 ± 1*	739 ± 16	38.6 ± 0.2
Oxotremorine	Control	178 ± 6	32 ± 9	8.4 ± 2	0.6 ± 0.3	205 ± 19	30.1 ± 0.2
	DFP	196 ± 7	50 ± 77	3.3 ± 0.7	0.6 ± 0.4	275 ± 28	31.6 ± 0.5

*Significantly different from control $P < 0.05$.

N = 12 for DFP treated.

N = 8 for Control

TABLE III

Measurements of Tolerance to DFP using

High Dose DFP Treatment

Challenge Drug	Group	Respiratory Rate (min^{-1})	Rotarod (sec)	Y-Maze Activity	Y-maze Rearings	Heart Rate bpm	Temperature (°C)
Nicotine	Control	320 ± 4	56 ± 6	0	0	473 ± 29	35.0 ± 0.1
(6)	DFP	290 ± 9	35 ± 6	0	0	488 ± 27	34.5 ± 0.1
Oxotremorine	Control	159 ± 11	13 ± 3	3 ± 1	1 ± 0.4	187 ± 19	30.9 ± 0.2
(6)	DFP	147 ± 11	15 ± 5	1 ± 0.4	0	242 ± 37	31.3 ± 0.1
Saline	Control	217 ± 25	55 ± 10	46 ± 5	25 ± 2	733 ± 10	38.5 ± 0.5
(12)	DFP	262 ± 17	69 ± 14	37 ± 6	18 ± 2	712 ± 16	38.5 ± 0.5
DFP	Control	134 ± 10	13 ± 3	4 ± 1	0	385 ± 45	32.3 ± 0.5
(12)	DFP	146 ± 20	22 ± 5	5 ± 1	0	278 ± 28	31.6 ± 1.0

Figure Legends

Figure 1. Time course of inhibition of brain acetylcholinesterase activity following a single intraperitoneal injection of DFP. Male DBA, C57BL and C3H mice were injected with DFP, 5.28 mg/kg, prepared in saline. At the times indicated, mice were killed and their brain acetylcholinesterase activity measured. Each point is the mean ± SEM of 4 to 6 determinations. Where not indicated, standard errors are contained within the plotted points. At all time points, brain acetylcholinesterase activity was significantly lower than control at time zero.

Figure 2. LD50 for DFP. Male (●) and female (○) DBA, C57BL and C3H mice were injected with various doses of DFP prepared in saline. Time of injection and death were noted, and the average time for death to occur is listed next to the data point in the body of the figure. Percent lethality was determined for 7 to 20 animals per point.

Figure 3. Effect of DFP on body temperature. Male (●) and female (○) DBA, C57BL and C3H mice were injected with DFP, 3.17, 4.22, 5.28 or 6.33 mg/kg (referred to as their integer values in the body of the figure) prepared in saline. Male (■) and female (□) controls were run along with the DFP-treated animals at each dose, but are plotted in only one panel for clarity. Each point is the mean ± SD of 8 animals per point. Standard deviation bars are directed down for males and up for females, except where obvious. Where not plotted, standard deviations are within the plotted point.

Figure 4. Effect of dose of DFP on brain acetylcholinesterase activity. Twenty-four hours after receiving 3.17, 4.22, 5.28 or 6.33 mg DFP/kg, brain acetylcholinesterase activity was measured. Each point is the mean ± SEM of 8 animals per point. Where not indicated, standard errors are contained within the plotted point. All doses of DFP significantly reduced brain AChE activity from control.

Figure 5. Relationship between maximum temperature depression (2 hours following DFP administration) and residual brain acetylcholinesterase activity 24 hours following DFP administration. Each of the slopes is significantly different from zero. Males (●) and females (○) have been plotted separately. There was no difference in the slopes of the male and female DBA and C3H strains. Slopes for the male and female C57BL mice were significantly different from each other, $p < 0.05$.

Figure 6. Rate of recovery of brain acetylcholinesterase activity following a single dose of DFP in male mice. Mice were injected with DFP (6.33 mg/kg) and the brain acetylcholinesterase activity was measured on the days indicated. Each point is the mean ± SEM of 6 to 12 mice per point.

Figure 7. Dose response relationship for return of brain acetylcholinesterase activity 13 days after a single injection of DFP. Various doses of DFP were given to male DBA mice. Each point is the mean ± SEM of 6 animals per point.

Figure 8. Effect of a second injection of DFP on rate of return of brain acetylcholinesterase activity in male DBA mice. On day 9 after one injection of DFP, mice were re-injected with 6.33 mg/kg DFP. Rate of recovery was measured as described in figure 6.

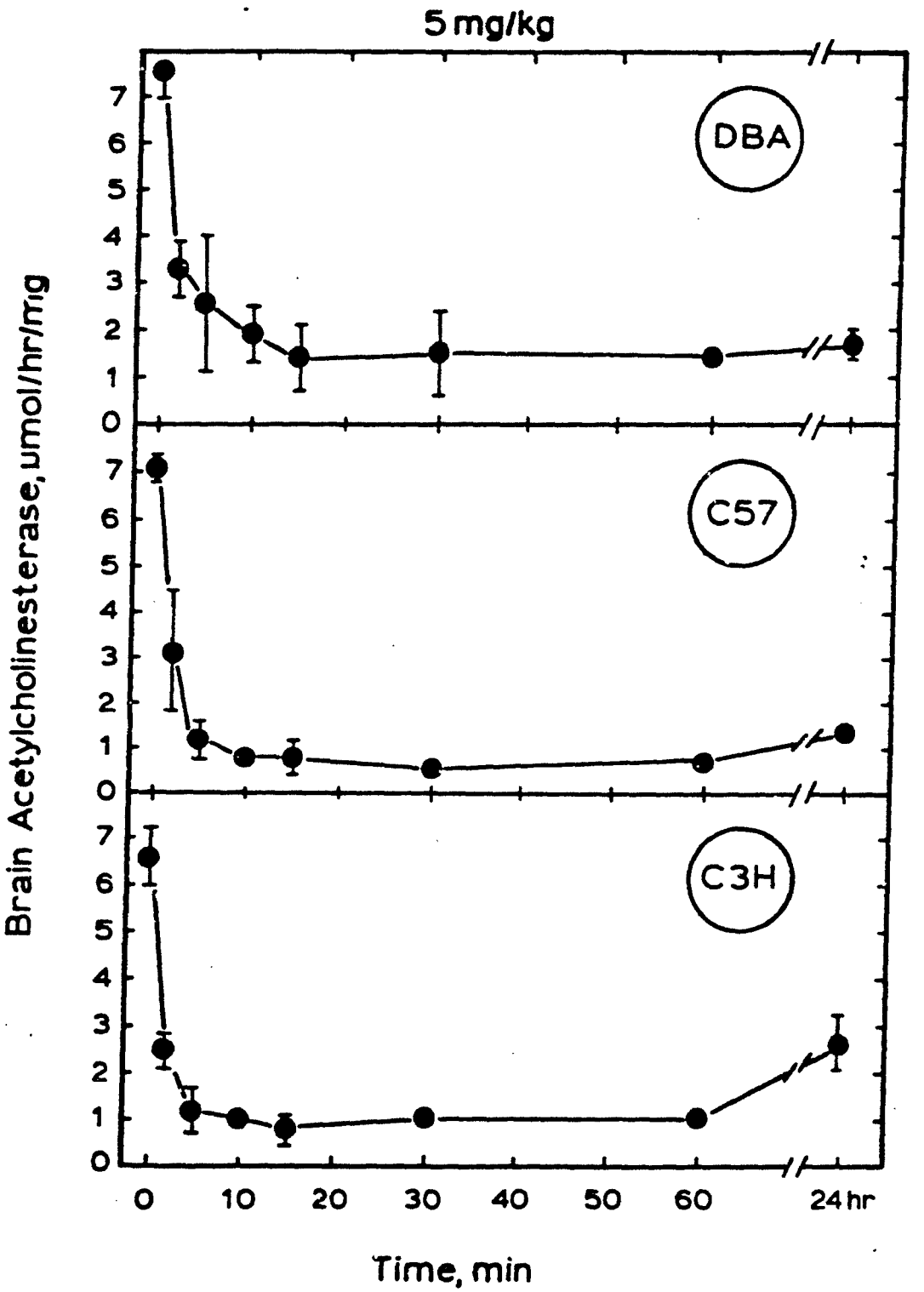
Figure 9. Rate of recovery of brain acetylcholinesterase activity following a single dose of DFP in female mice. Conditions were the same as described in figure 6.

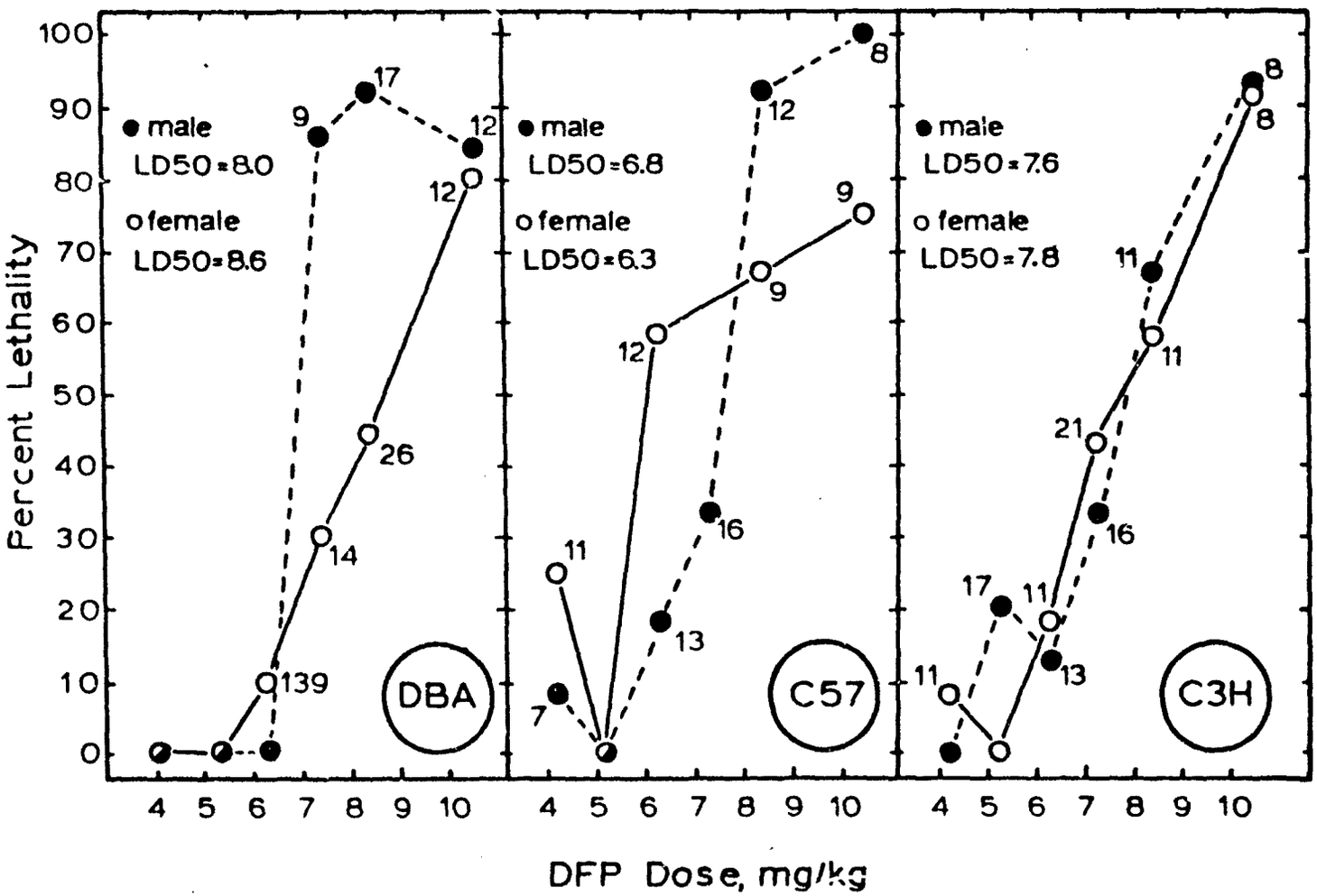
Figure 10. Effect of repeated testing with the test battery. The values are mean ± SEM of 6 animals per point. ( ) saline-treated 3 consecutive days (group 7); ( ) saline-treated; ( ) nicotine-treated; ( ) oxotremorine-treated. For example, Day 1 nicotine ( ) are pooled data of groups 3 & 4 (see text), Day 2 nicotine are data of groups 1 & 5, and Day 3 nicotine are data of groups 2 & 6.

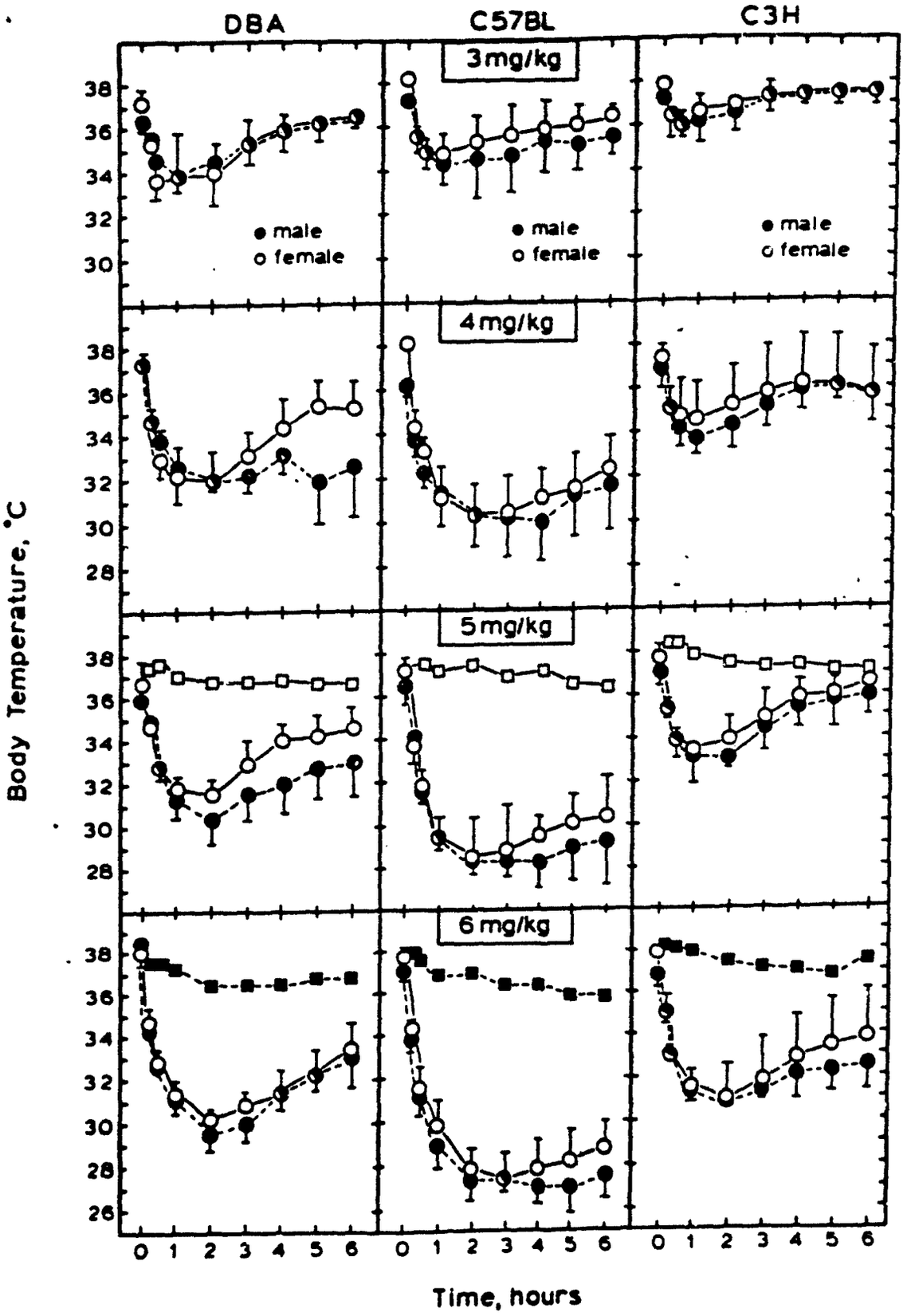
Figure 11. Dose response curve and time couse for the effect of DFP on heart and respiratory rates. Six to ten male DBA mice were injected with various doses of DFP and their response were monitored as a function of time. Separate goups were used for each measure. Each point is the mean ± SEM.

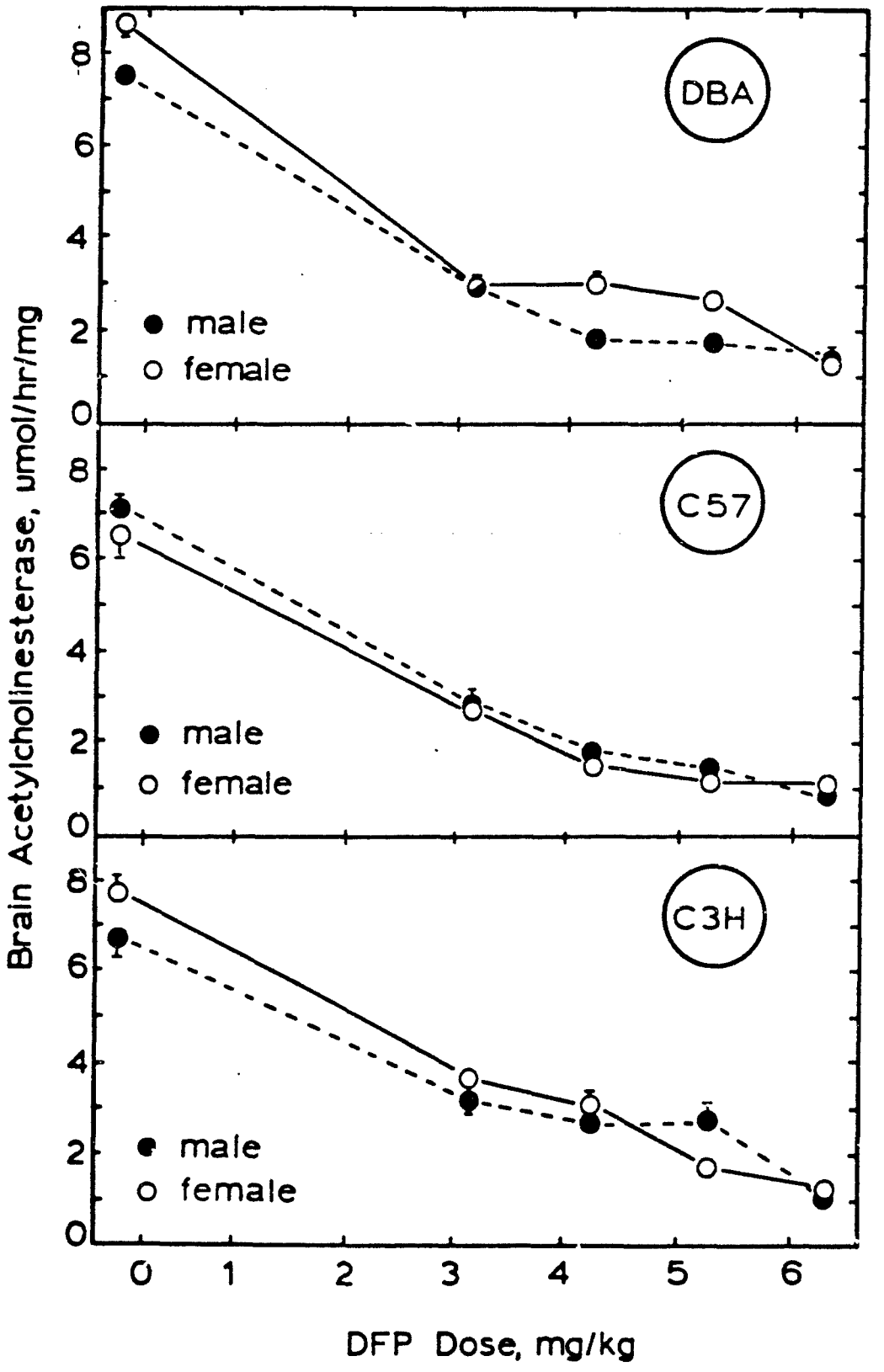
Figure 12. Effect of DFP on activity measurements. Male DBA mice were injected with various doses of DFP and their Y-maze and rotarod scores were measured as a function of time. Each point is the mean ± SEM of 6 to 12 animals per point. Separate groups were used for Y-maze and rotarod measurements.

Figure 13. The effect of chronic treatment with DFP on test battery performance. The reader is referred to the text for a summary of this figure. Open bars are controls (saline) and closed bars are DFP-treated. The dotted line represents the performance of naive, untreated controls.

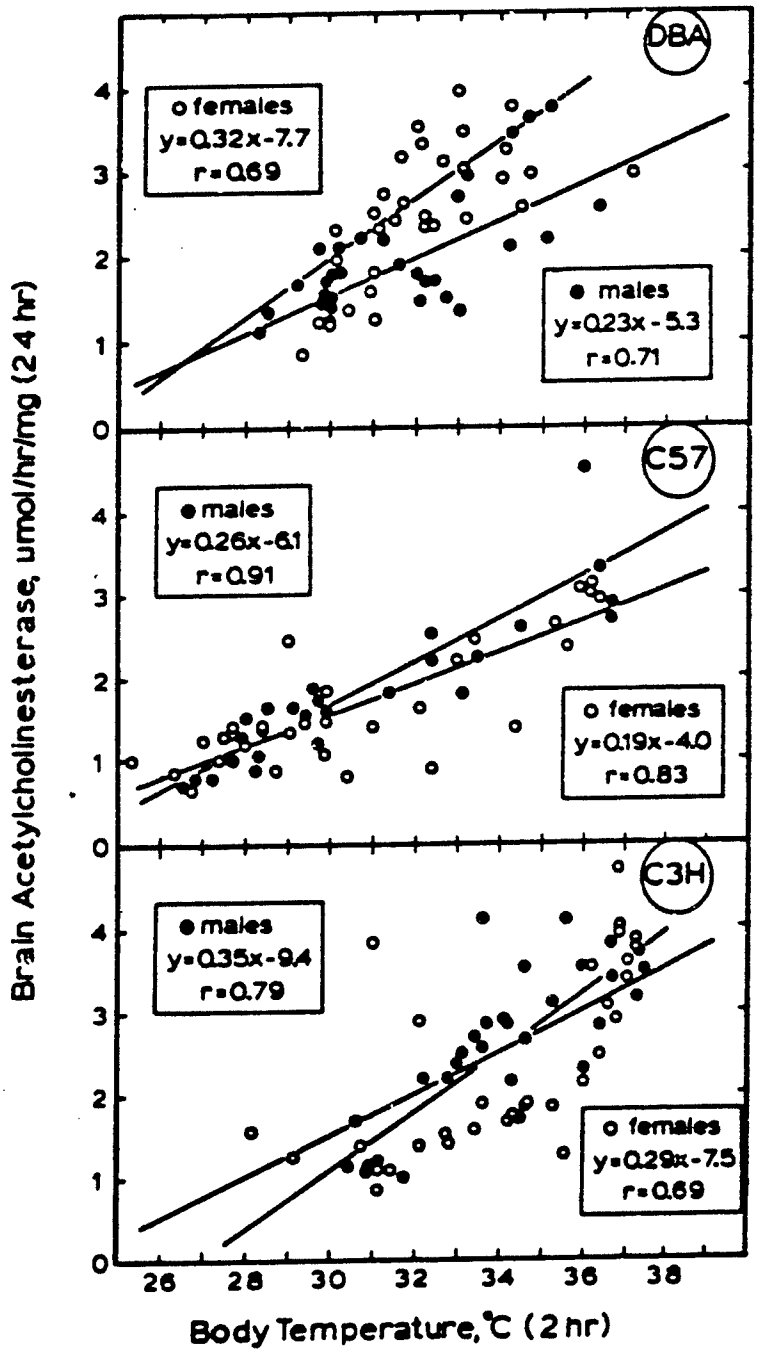

5 mg/kg
DBA
C57
C3H
Brain Acetylcholinesterase, umol/hr/mg
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
24 hr
Time, min

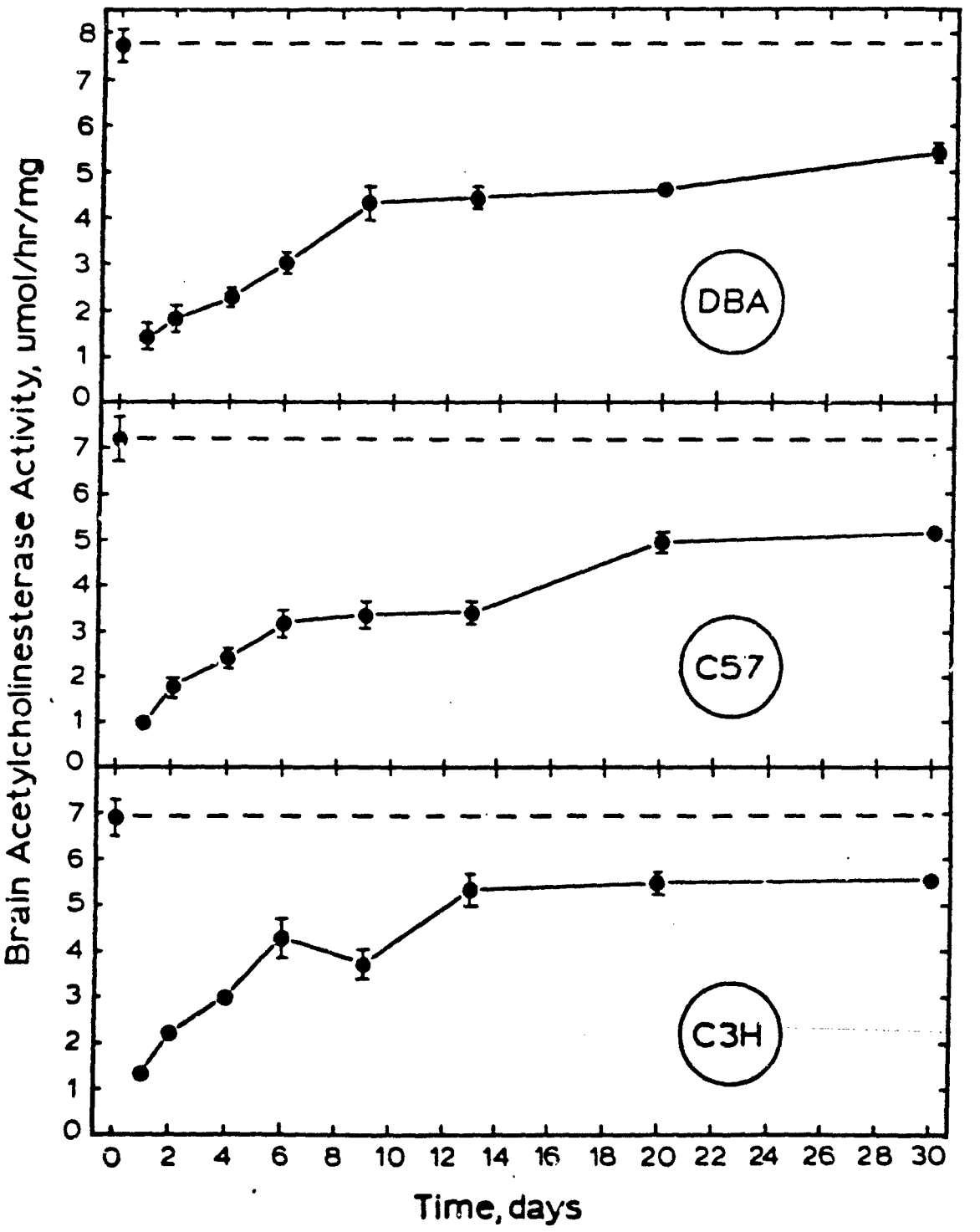

Percent Lethality
DFP Dose, mg/kg
male
LD50=8.0
female
LD50=8.6
DBA
male
LD50=6.8
female
LD50=6.3
C57
male
LD50=7.6
female
LD50=7.8
C3H

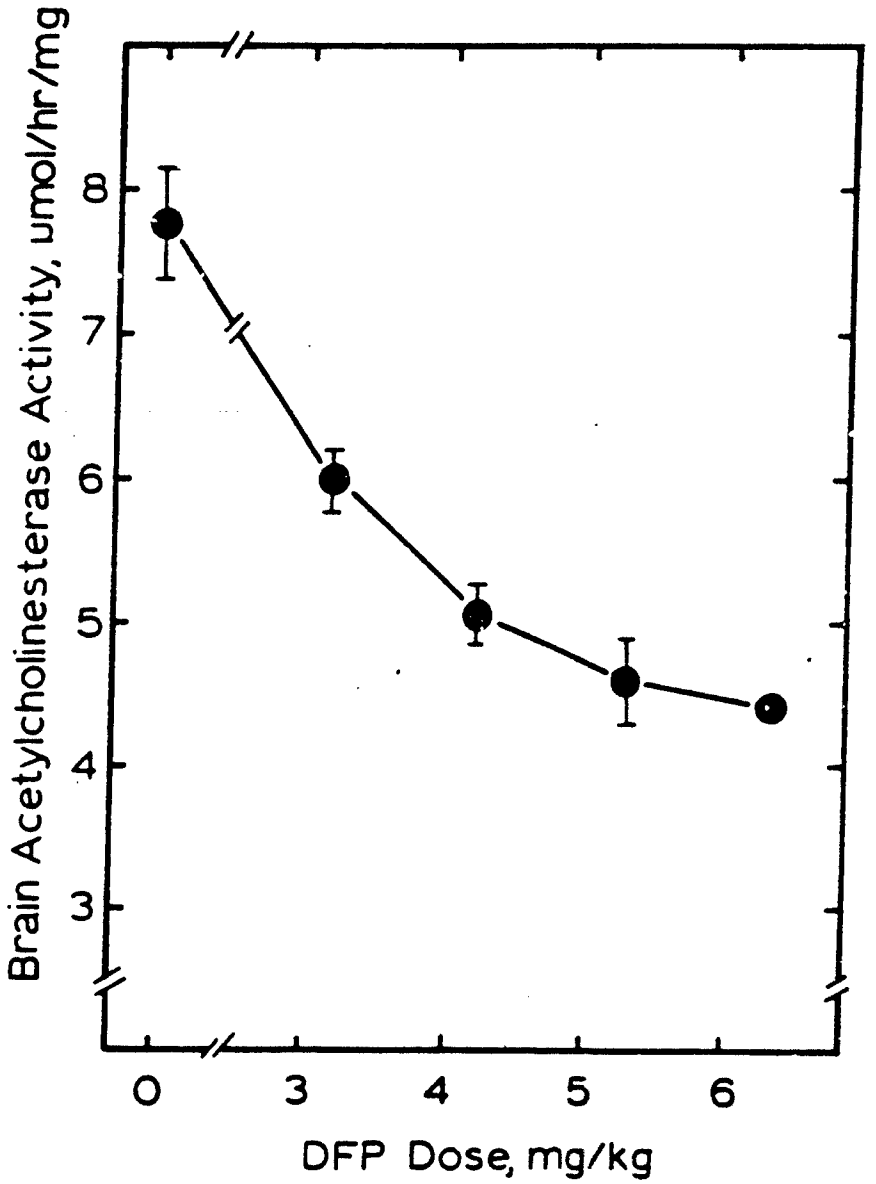

DBA
C57BL
C3H
3mg/kg
4mg/kg
5mg/kg
6mg/kg
male
female
Body Temperature, °C
Time, hours

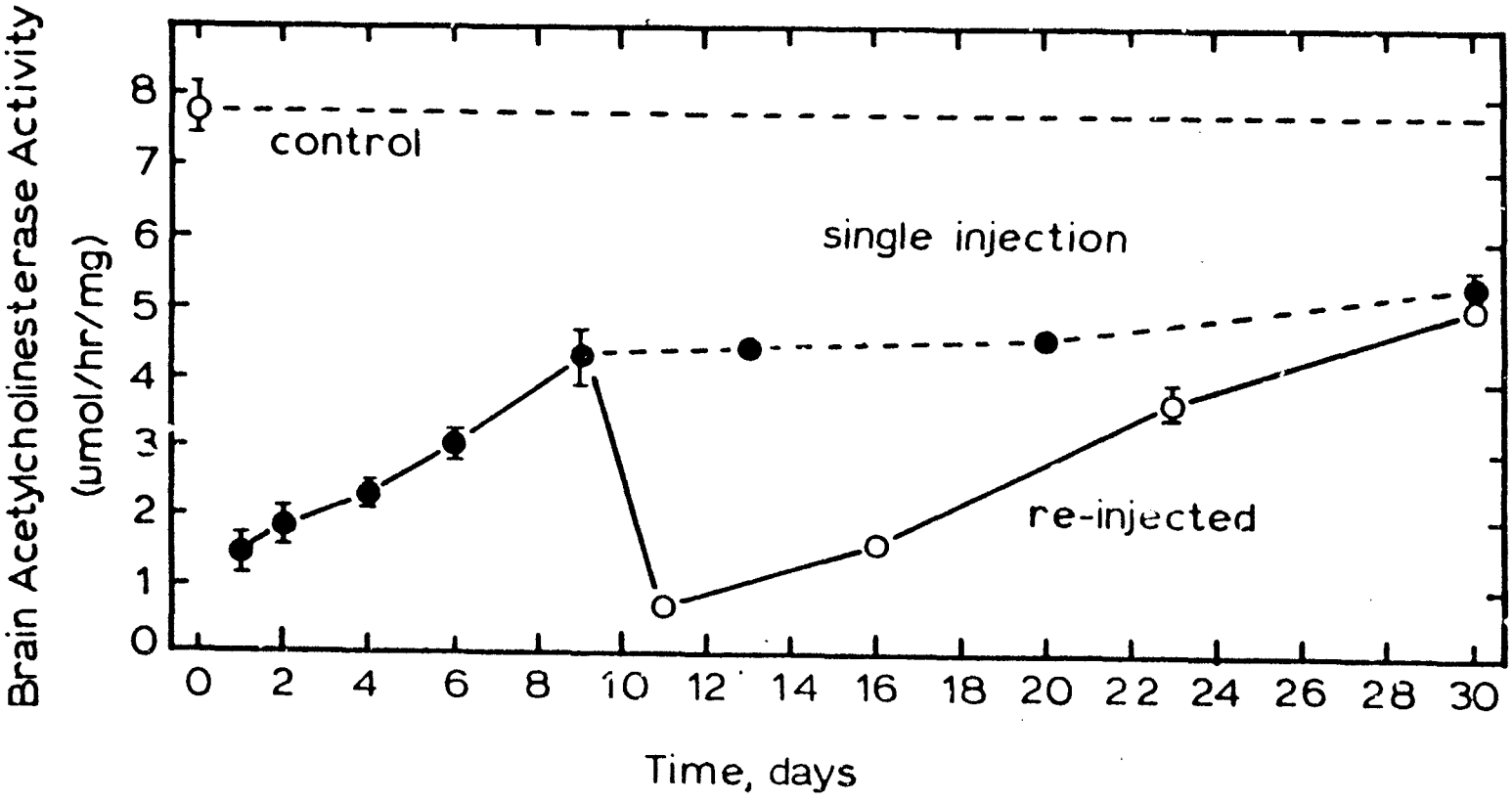

DBA
C57
C3H
male
female
Brain Acetylcholinesterase, umol/hr/mg
DFP Dose, mg/kg
0
2
4
6
8
0
1
2
3
4
5
6

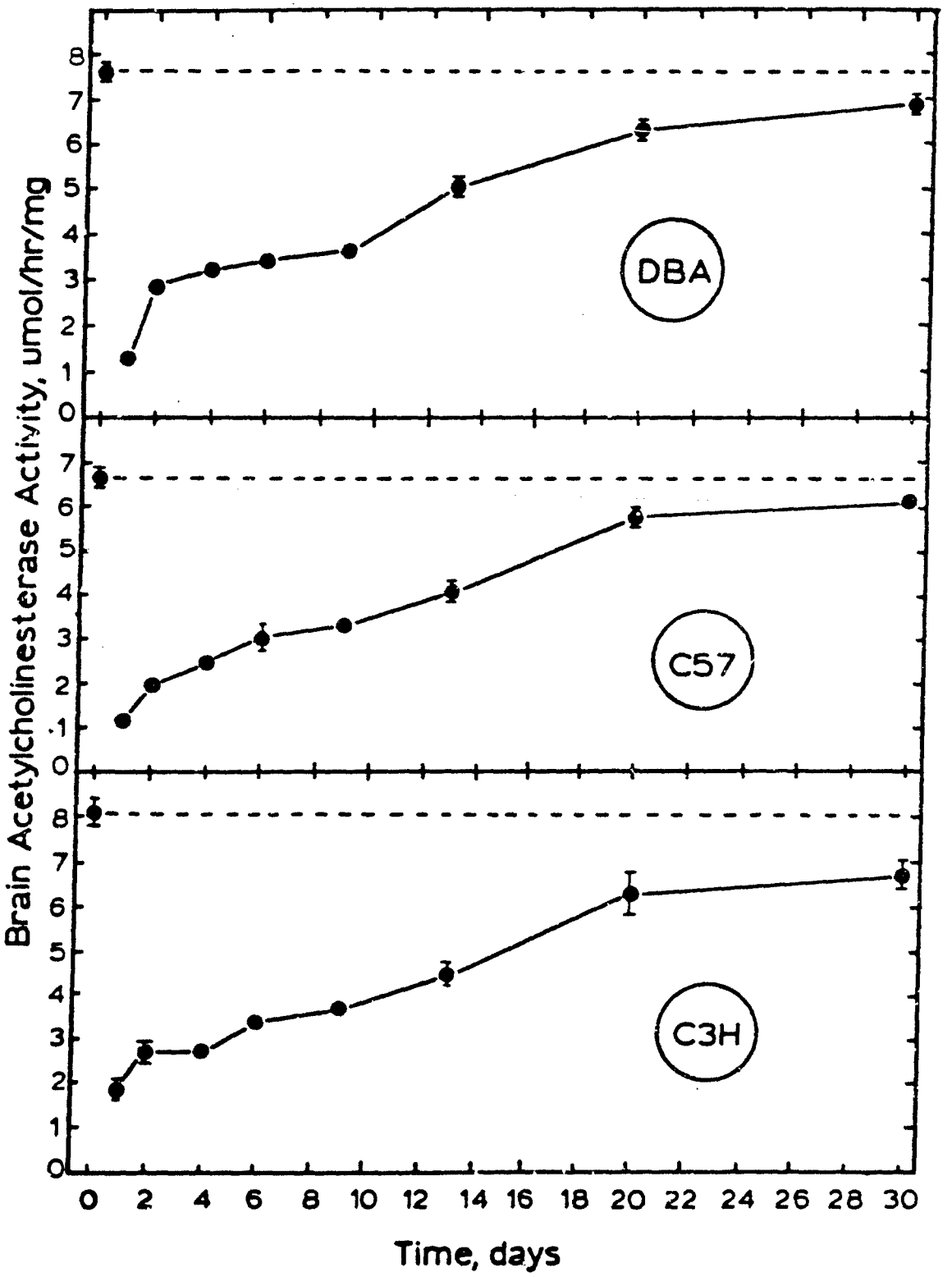
Temperature vs AChE Activity

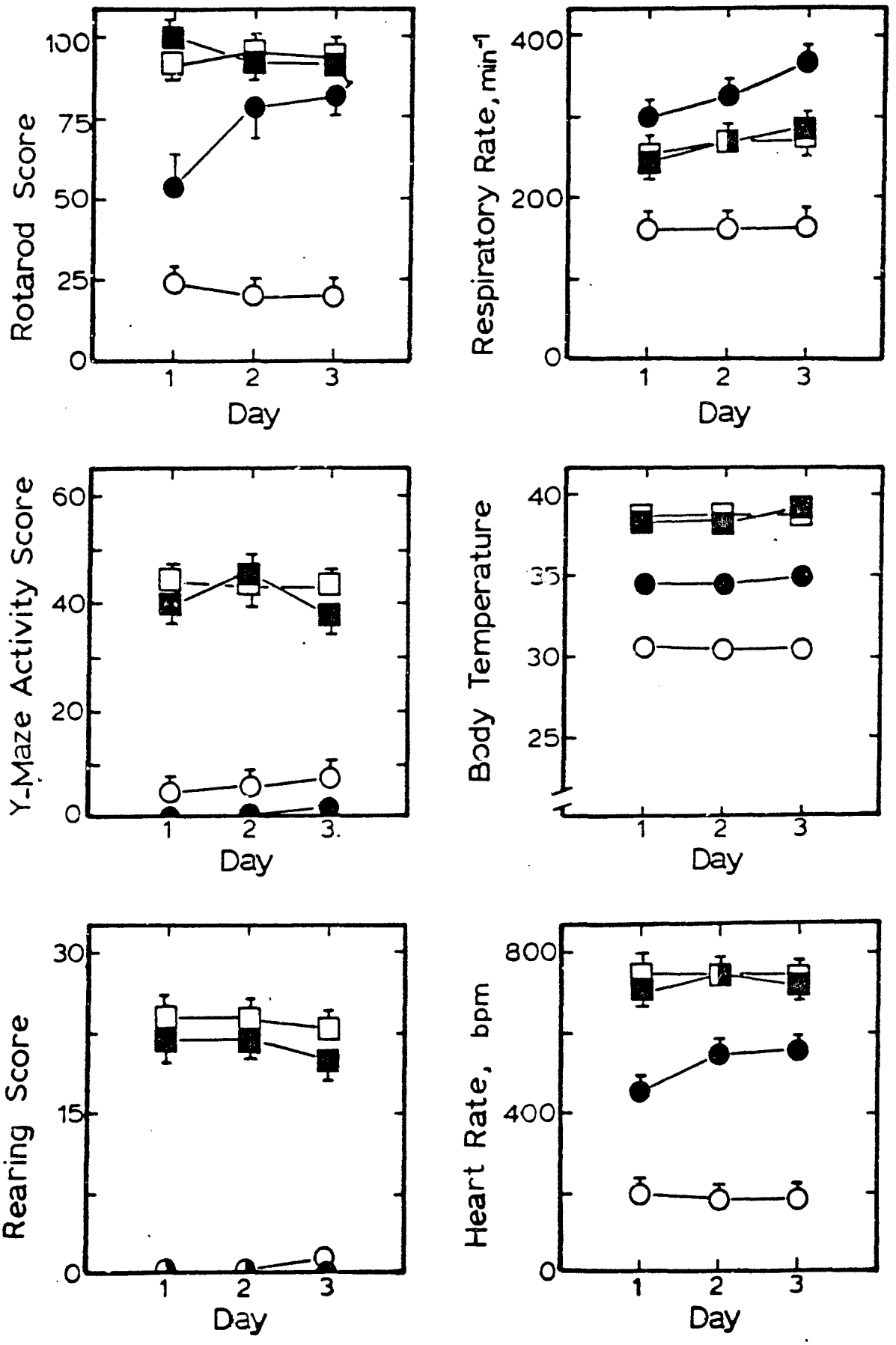


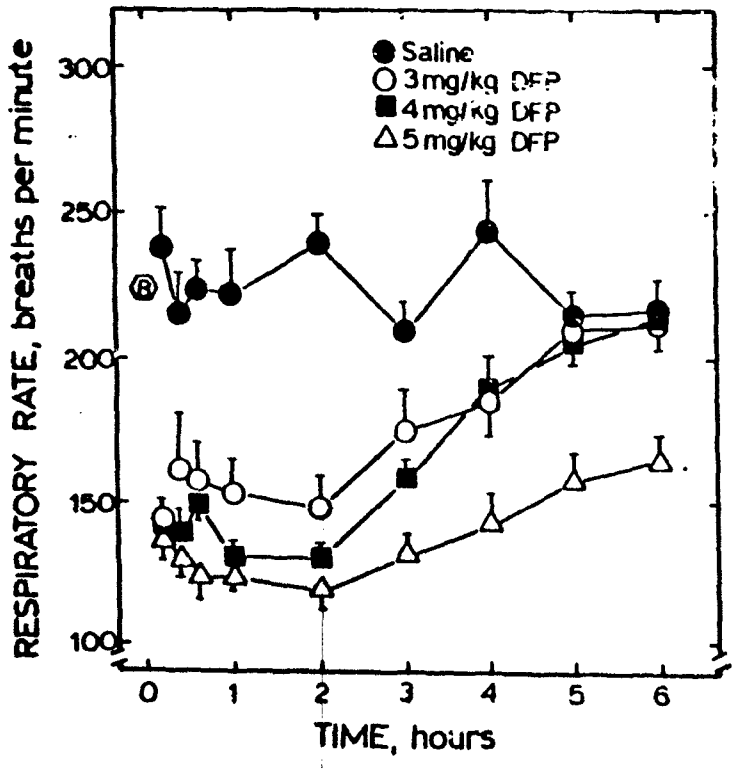

Brain Acetylcholinesterase Activity, umol/hr/mg
DBA
C57
C3H
0 1 2 3 4 5 6 7 8
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time, days

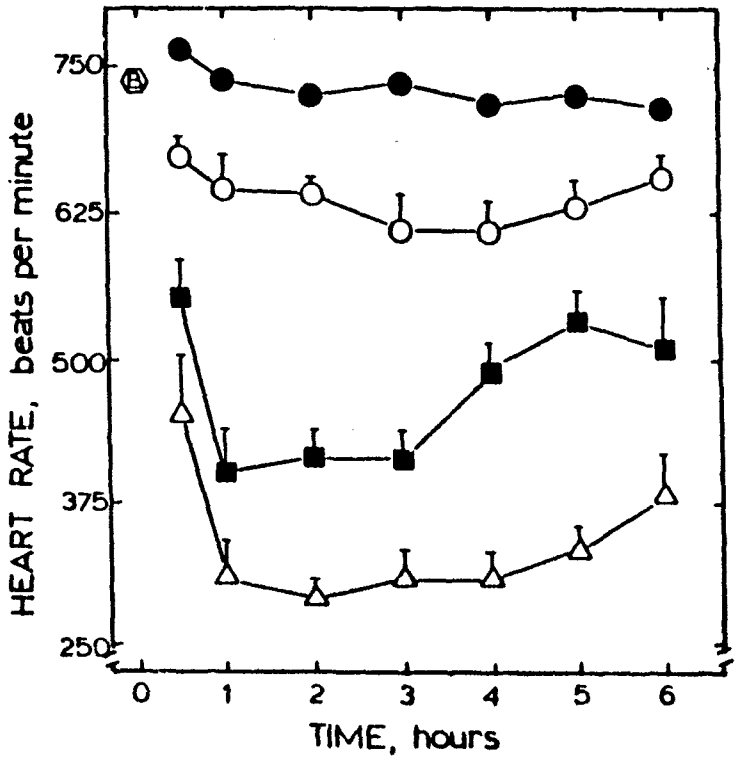

Brain Acetylcholinesterase Activity, umol/hr/mg
8
7
6
5
4
3
0
3
4
5
6
DFP Dose, mg/kg

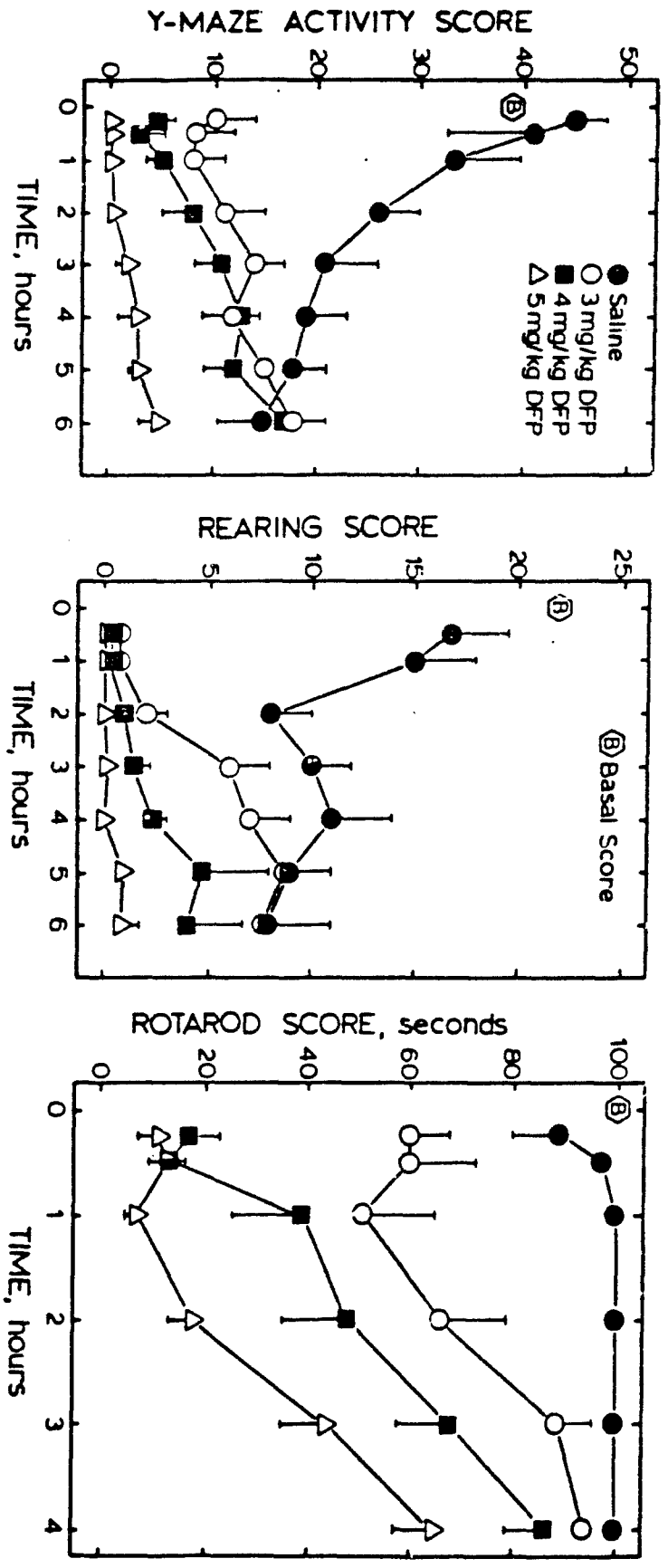

Brain Acetylcholinesterase Activity
(umol/hr/mg)
control
single injection
re-injected
0
1
2
3
4
5
6
7
8
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time, days

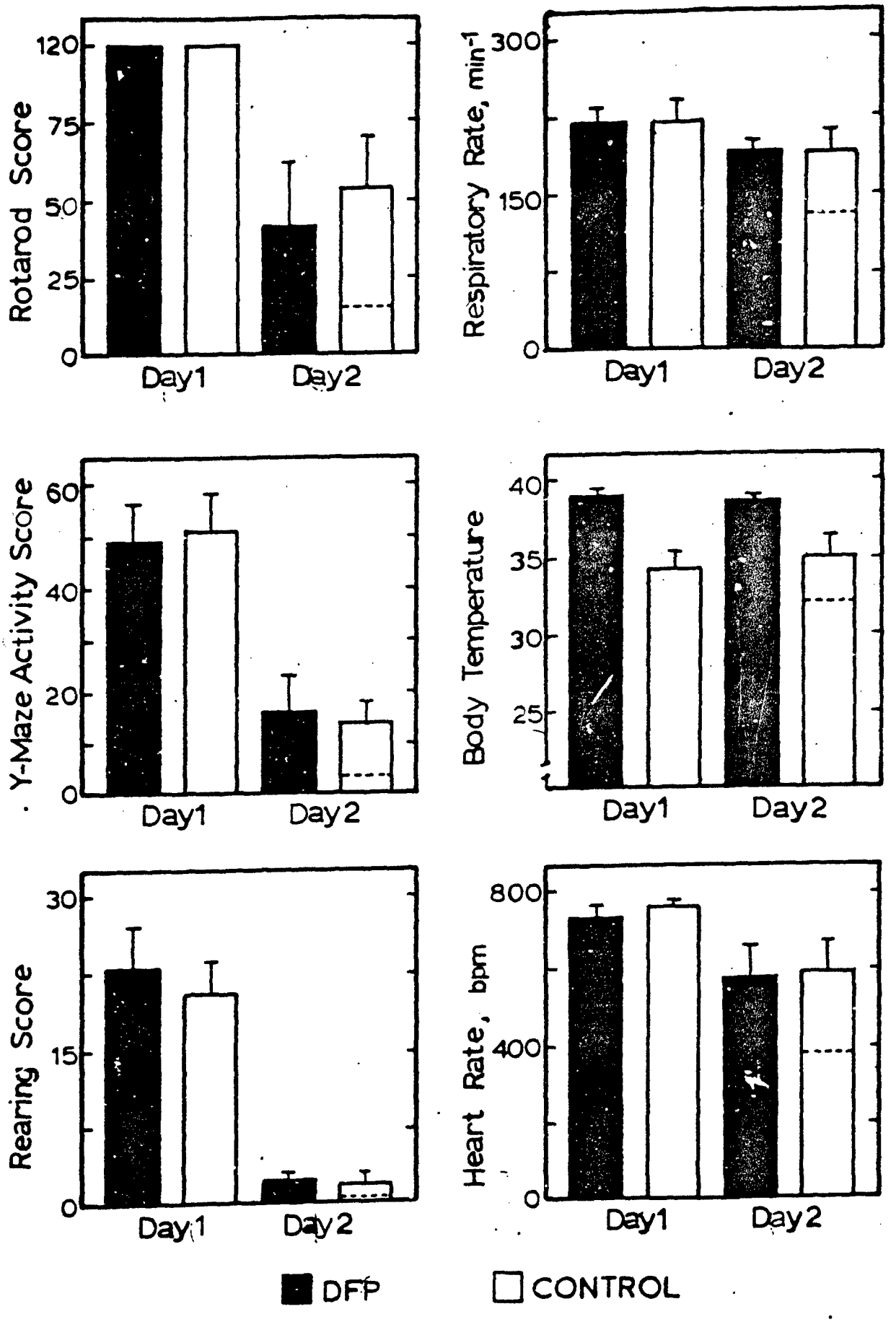

Brain Acetylcholinesterase Activity, umol/hr/mg
DBA
C57
C3H
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time, days


Rotarod Score
100
75
50
25
0
1
2
3
Day
Respiratory Rate, min⁻¹
400
200
0
1
2
3
Day
Y-Maze Activity Score
60
40
20
0
1
2
3
Day
Body Temperature
40
30
25
1
2
3
Day
Rearing Score
30
15
0
1
2
3
Day
Heart Rate, bpm
800
400
0
1
2
3
Day


Saline
3 mg/kg DFP
4 mg/kg DFP
5 mg/kg DFP
RESPIRATORY RATE, breaths per minute
300
250
200
150
100
0
1
2
3
4
5
6
TIME, hours


HEART RATE, beats per minute
750
625
500
375
250
0
1
2
3
4
5
6
TIME, hours


Y-MAZE ACTIVITY SCORE
0
10
20
30
40
50
TIME, hours
0
1
2
3
4
5
6
Saline
3 mg/kg DFP
4 mg/kg DFP
5 mg/kg DFP
B
REARING SCORE
0
5
10
15
20
25
TIME, hours
0
1
2
3
4
5
6
B
Basal Score
ROTAROD SCORE, seconds
0
20
40
60
80
100
TIME, hours
0
1
2
3
4
B


Rotarod Score
120
75
50
25
0
Day1
Day2
Respiratory Rate, min-1
300
150
0
Day1
Day2
Y-Maze Activity Score
60
40
20
0
Day1
Day2
Body Temperature
40
35
30
25
Day1
Day2
Rearing Score
30
15
0
Day1
Day2
Heart Rate, bpm
800
400
0
Day1
Day2
DFP
CONTROL