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Effects of Anisomycin on Retention of the
Passive-Avoidance Habit as a Function of Age

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Abstract

Three age groups of male Swiss albino CD-1 mice (2-3 mo, 6-7 mo, and 14-15 mo) were treated with a 120 mg/kg dose of the protein synthesis inhibitor anisomycin or with an equal volume of saline at various times before and after training (20 min pretraining, 0, 10, 30, and 180 min posttraining) in a shock motivated passive-avoidance task. Young (2-3 mo) and intermediate-aged (6-7 mo) mice treated with anisomycin before or immediately after training demonstrated impaired retention at a 7 day test, but retention was normal for mice injected 10, 30, or 180 min posttraining. The older mice (14-15 mo) showed similar results, with one exception: Those older mice injected with anisomycin 10 min posttraining were significantly impaired in retention as compared to older saline controls and to identically treated young or intermediate-aged mice. The prolonged gradient of retrograde amnesia demonstrated by older mice could not be accounted for by impaired acquisition, impaired short-term memory, altered spontaneous locomotor activity, or differential inhibition of brain protein synthesis.

active-avoidance, aging, amnesia, anisomycin, locomotor activity, memory, passive-avoidance, protein synthesis inhibition, T-maze

Protein synthesis has been postulated to play an important role in both memory formation [17] and in aging [13]. In the case of memory, numerous studies have demonstrated that antibiotic drug-induced inhibition of cerebral protein synthesis shortly before or after training markedly impairs long-term retention in a variety of tasks and species [3, 15]. Acquisition and short-term memory are normal [2, Note 1]. The usual interpretation of these studies is that cerebral protein synthesis inhibitors produce amnesia by blocking the synthesis of proteins specifically required for the formation of long-term memory, and that acquisition and short-term memory are independent of brain protein synthesis. Various alternative hypotheses have been considered in detail previously and ruled out [6 and references therein]. Studies of protein synthesis alterations as a function of age have primarily concentrated on protein content and/or synthesis rates, changes in amino acid sequence, and alteration in posttranslational reactions. The rate of protein synthesis in various brain regions [11] as well as transport of proteins to specific brain fractions [1], as indicated by radioactive incorporation studies, has been reported to decrease as a function of age. Additionally, alterations in the content of the brain-specific proteins S100 and 14-3-2 during aging have been reported [5]. Considering the prominence that protein synthesis is hypothesized to play in the pharmacology of memory and biology of aging, it is somewhat surprising that no attempts have been made to investigate a possible relationship between changes in protein synthesis efficiency and memory.

In this study we consider the possibility that a protein synthesis inhibitor, anisomycin (ANI), might demonstrate differential effects on memory as a function of age. We have examined this possibility by treating three age groups of mice (2-3 mo--young, 6-7 mo--intermediate, and 14-15 mo--older) with

ANI or saline at various times before and after training in a shock motivated passive-avoidance task. We arbitrarily refer to the different age groups of mice throughout this study as "young", "intermediate", and "older". The meaning of these designations is restricted to information about the relative ages of these groups in relation to one another.

Method

Subjects: The subjects were male Swiss Webster CD-1 mice from the Charles River Breeding Laboratories. Mice were grouped according to three ages: 2-3 month (young), 6-7 month (intermediate), and 14-15 months (older). Young and intermediate age mice were received at 55 days of age and older mice were received at 6 months of age. Animals were housed 4-5 to a cage until 48 hr prior to training when they were housed individually and remained so throughout the experiment. Mice were maintained on a 12-12 light-dark cycle and with ad lib access to food and water.

Passive-Avoidance Apparatus and Procedure: Mice received one-trial passive avoidance training in a step-through apparatus described previously [7]. Briefly, the apparatus was constructed of Plexiglas. A black panel with a 3.8 cm dia. hole at the base separated a black start box (9 cm long x 10.2 cm wide x 12.5 cm high) from a white shock compartment (35 cm long x 8.2 cm wide x 12.5 cm high). The apparatus was illuminated by a 1.8 W light bulb situated behind a white translucent panel at the end of the shock compartment. A white translucent guillotine door blocked access to the shock compartment prior to

training. A 0.30 mA shock was delivered through 2.4 mm dia. brass rods in the shock compartment by a constant current 18-pole shock scrambler. The apparatus was wiped clean with alcohol and allowed to dry between the testing of successive animals.

For training, a mouse was placed into the start box for 10 sec, then the light illuminating the apparatus was turned on. Ten seconds later the guillotine door was removed when the mouse was oriented away from the entrance. The step-through-latency (STL) was measured as the time from orientation to the entrance until the animal had all four paws on the shock grid. Five seconds later footshock was delivered until the mouse escaped back to the start box. The guillotine door was replaced and the light turned off. After 5 sec the mouse was returned to its home cage until retention testing 7 days later. Retention testing was identical except that no footshock was delivered.

Drug: ANI (2-p-methoxyphenyl-3-acetoxy-4-hydroxypyrrolidine) was dissolved in saline by adding an approximately equal molar amount of 3N HCl and adjusting the pH to 6-7 with NaOH. Subcutaneous injections of saline or the saline solution containing ANI (12 mg/ml) were made on the back of mice either 20 min prior to training, immediately after training, 10 min, 30 min, or 180 min after training in a dosage of 120 mg/kg.

Results and Discussion

The mean training STLs for mice receiving posttraining treatments were 7.6 ± 0.3 , 7.2 ± 0.3 , and 11.6 ± 0.4 for young intermediate, and older mice respectively, and a one-way analysis of variance revealed a significant effect

of age, $F(2,406) = 42.4$, $p < 0.001$. Application of the Scheffé procedure at the 0.05 level indicated this effect was due to the greater STLs of older mice as compared to either young or intermediate age mice. The mean escape latencies for young, intermediate, and older mice were 4.5 ± 0.3 , 5.0 ± 0.3 , and 5.7 ± 0.3 respectively, and a significant effect of age was due to the difference between young and older mice, $F(2,406) = 4.2$, $p < 0.025$. Analysis of pre-training injections revealed a significant effect of ANI on escape latencies in young mice, $F(1,34) = 15.0$, $p < 0.001$, and STLs in intermediate age mice, $F(1,28) = 7.5$, $p < 0.025$. It has been demonstrated previously that an increase in STLs and/or escape latencies results in greater training strengths [10]. Since in this experiment ANI treated mice, in all cases, demonstrated higher STLs and escape latencies at training than corresponding saline controls, the amnesic effect of this agent cannot be explained in terms of differing training strengths.

The median STLs shown at a 7-day retention test by ANI treated mice are given in Figure 1. For each age group and injection time, a corresponding saline control group exhibited a median STL of 300 sec. Irrespective of age, mice treated with ANI 20 min prior to training or immediately after training demonstrated impaired performance at test. Young and intermediate age mice that were injected with ANI 10 min after training were not significantly different from saline controls, but older mice injected 10 min after training showed significantly impaired performance at test as compared to older mice injected with saline at an identical time ($p < 0.001$). Furthermore, older mice treated with ANI 10 min posttraining were significantly impaired at test as compared to young and intermediate age mice treated with ANI at the same time ($p < 0.05$). All age groups of mice treated with ANI 30 or 180 min posttraining

had normal retention performance at test 7 days later,

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(Insert Figure 1 about here)
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The finding that the retrograde amnesic gradient of ANI is extended in older mice suggests that the transition from protein synthesis independent short-term memory to protein synthesis dependent long-term memory [2, 16] may be slower in older mice than young or intermediate age mice. Alternative hypotheses such as impaired acquisition, impaired short-term memory, or differential drug effectiveness are easily conceivable and are examined in subsequent experiments.

Experiment 2

In this experiment we examine the possibility that older mice have poorer acquisition of passive-avoidance training than young or intermediate age mice. If there is differential acquisition of the passive-avoidance habit as a function of age then it should be possible to demonstrate differential test performance shortly after training. To test this possibility we examined retention 30 min after training. Choice of test time was based on the observation that ANI injected 30 min after training did not differentially affect retention of different aged mice (see Exp. 1).

Method

The behavioral apparatus, training, and subjects were the same as in Experiment 1. The differences in procedure were that mice received no injection and that retention testing was conducted 30 min after training.

Results and Discussion

The median STLs for young ($N=20$), intermediate ($N=20$), and older mice ($N=14$) were 89.5, 112.5 and 143.5 respectively, and there was no significant difference between any groups ($p > 0.25$ in all cases). Thus, differential acquisition does not appear to be able to account for the prolonged retrograde amnesia gradient in older mice treated with ANI.

Experiment 3

In this experiment we examined the possibility that the prolonged susceptibility to the amnesic action of ANI by older mice might be due to impaired short-term memory. While the results from passive-avoidance tests at 30 min after training appear contrary to this possibility, it still seemed important to further test this possibility by utilizing a task that presumably requires extensive use of short-term memory. For this reason, we examined acquisition of a multiple-trial left-right discrimination in a T-maze by young, intermediate, and older mice.

Method

The training apparatus was a Plexiglas T-maze (12.5 cm high, 9.8 cm wide throughout, the stem being 46 cm long, and each arm 17.5 cm long) painted flat black except for a clear top. A black Plexiglas guillotine door 11 cm from the closed end of the stem formed a start box and prevented the mouse from moving down the start alley prior to the beginning of each trial. Each arm of the maze was lined with a removable clear Plexiglas container that was used for removing animals from the maze after each trial; the container bottom extended below the shock grid. Footshock (0.30 mA) was delivered through 2.4 mm dia. brass rods by an 18 pole shock scrambler.

Training began by placing each mouse in the start box. After 5 sec a loud door bell buzzer sounded and the guillotine door was removed. Five seconds later footshock was initiated and continued until the mouse entered the correct arm of the maze. On the first trial the initial entrance into a maze arm was considered incorrect and the buzzer and shock continued until the mouse moved into the other alley. On subsequent trials the arm initially entered on the first trial was incorrect and the opposite arm correct. If the mouse entered the correct alley prior to shock onset, the buzzer was turned off and an avoidance response was scored. When the mouse entered the correct alley after shock onset the buzzer and shock were terminated and an escape response was scored. The alley entrance was blocked with a guillotine door immediately after a correct response and 10 sec later the mouse was returned to its home cage by lifting the Plexiglas liner out and placing it in the animal's home cage. If the mouse did not leave the liner after 5-10 sec, a gentle touch to the hindquarters encouraged the mouse to return to its home cage. The intertrial interval was approximately 30 sec. Training continued

for 34 trials, not including trial 1, or until the mouse made 4 correct avoidance responses within 5 consecutive trials.

Results and Discussion

The numbers of mice reaching a criterion of 4 out of 5 avoidance responses within 34 trials were 12, 12, and 10 out of an N of 15 for young, intermediate, and older mice respectively [$X^2(2)=1.1$, $p>0.50$]. The mean numbers of trials to criterion for those mice reaching criterion within 34 trials were 19.5 ± 2.4 , 17.2 ± 1.9 , and 18.0 ± 1.9 for young, intermediate, and older mice respectively. A one-way analysis of variance indicated no significant difference between groups, $F(2,31)=.3$, $p>0.70$. Thus, normal performance of a left-right discrimination in a T-maze and normal retention shortly after passive-avoidance training by older mice indicate that their prolonged susceptibility to the amnesic action of ANI cannot be accounted for in terms of an impaired short-term memory.

Experiment 4

Performance in the passive-avoidance task may be confounded by treatments or conditions that affect spontaneous locomotor activity [8]. Therefore, in this experiment we examine the locomotor activity of mice as a function of age.

Method

Spontaneous locomotor activity was measured automatically in a Plexiglas box (30.5 cm square x 15.5 cm high) painted flat black except for a clear top. The activity box was divided into quadrants by photocells. A mouse was placed into the activity box and the number of quadrant crossings per minute for 5 min were automatically recorded. The activity box was cleaned with alcohol and allowed to dry between the testing of successive mice.

Results and Discussion

The mean number of crossings per minute during the 5 minute session were 28.2, 25.5 and 21.7 for young, intermediate, and older mice respectively. A two-way analysis of variance with repeated measures revealed that age significantly affected activity, $F(2,42)=4.6$, $p<0.025$. Application of the Scheffé procedure at the 0.05 level indicated this effect was due to the difference between young and older animals. Activity significantly decreased over time [$F(4,168)=52.4$, $p<0.001$], and there was no interaction between age and time, $F(8,168)=.9$, $p>0.50$.

The lower spontaneous locomotor activity scores demonstrated by older mice would predict good passive-avoidance performance since this task requires the suppression of a locomotor response. Thus, the prolonged retrograde amnesic gradient of ANI in older mice cannot be due to differential activity levels demonstrated by the different age groups of mice.

Experiment 5

Age can significantly affect such pharmacokinetic parameters of drugs as absorption, distribution, and metabolism. It is therefore possible that the differential behavioral effects of ANI (Exp. 1) might reflect age-induced differential effectiveness of ANI. To investigate this possibility, we examined the effects of a subcutaneous injection of ANI (120 mg/kg) on whole brain protein synthesis. We choose this biochemical action of ANI for investigation because previous behavioral studies have shown that a subcutaneously administered protein synthesis inhibitor must inhibit whole brain protein synthesis by approximately 80-90% in order to produce amnesia [3].

Method

The method for evaluating protein synthesis inhibition has been described in detail previously [7]. Briefly, CD-1 mice were injected subcutaneously with L-[U-¹⁴C] valine at various times after administration of ANI (120 mg/kg) or saline, and they were then sacrificed 20 min later by cervical dislocation. Protein synthesis during the period of radioactive incorporation was calculated by determining the ratio of 1) radioactivity resulting from incorporation of label into trichloroacetic acid insoluble material to 2) total radioactivity in the entire brain sample. The ratio derived from ANI treated mice was compared to the ratio of saline treated mice in calculating percentage inhibition.

Results and Discussion

Determination of percent inhibition produced by ANI in the different age mice is shown in Figure 2. A two-way analysis of variance revealed no significant effect of age on ANI-induced inhibition of whole brain protein synthesis between 20 min and 5 hr after injection, $F(2,9)=.15$, $p>0.80$. The time course of evaluation extended to a point (5 hr) beyond which inhibition fell below the level required for inducing amnesia (80-90%). Thus, neither differential extent or duration of brain protein synthesis inhibition can account for the amnesia demonstrated by older mice injected with ANI 10 min after passive avoidance training.

(Insert Figure 2 about here)

General Discussion

These experiments indicate that the retention of older mice is susceptible to disruption by the amnesic agent ANI for a longer time after training than normally occurs in young or intermediate age mice. This prolonged retroactive amnesic gradient does not appear to be due to impaired acquisition, impaired short-term memory, or altered spontaneous locomotor activity in older mice. Furthermore, the effectiveness of ANI 10 min posttraining in older mice as compared to young or intermediate age mice was not due to differential inhibition of whole brain protein synthesis. Our preliminary interpretation of these results is that the transition time from short-term to

long-term memory is longer in older mice than in younger mice. By this view, during or shortly after learning short-term processes initiate synthesis of proteins required for the establishment of long-term memory. If this process is slower in older mice, then ANI would have an extended period during which it could block protein synthesis prior to its reaching some threshold required for establishment of long-term memory. This suggestion is consistent with the hypothesis, based on numerous pharmacological studies of learning and memory, that protein synthesis inhibitors impair long-term memory by blocking the synthesis of proteins specifically required for the establishment of memory.

In this study we examined the biochemical effect of ANI on whole brain protein synthesis. It would be of considerable interest in future studies to examine the relationship between protein synthesis in particular fractions of brain and learning and memory. This strategy has been attempted in younger animals, but no amnesia was produced unless whole brain protein synthesis inhibition exceeded 80-90% [12]. Older animals might offer a model for investigating this relationship since it has been reported previously that there is a reduction in the incorporation of $[1-^{14}\text{C}]$ leucine into the synaptosomal brain fraction of 12 mo old mice as compared to 3 mo old mice [1]. This suggests that transport of protein to nerve endings decreases with age. Future research might be directed at determining whether there is a relationship between the age-associated decrease in incorporation of a radioactively labeled amino acid into the synaptosomal fraction and age-related decrements in memory.

Finally, protein synthesis studies may have implications for studies focusing on cholinergic alteration as a function of age. It has been frequently suggested that changes in the cholinergic system might play a significant role in age associated memory decrements [4, 9, 17]. A possible interaction between the cholinergic system and protein synthesis is suggested by the

report that the protein synthesis inhibitor cycloheximide blocks increased QNB binding at 30 min after passive avoidance training [14]. Further research on this relationship needs to be pursued in both young and old mice.

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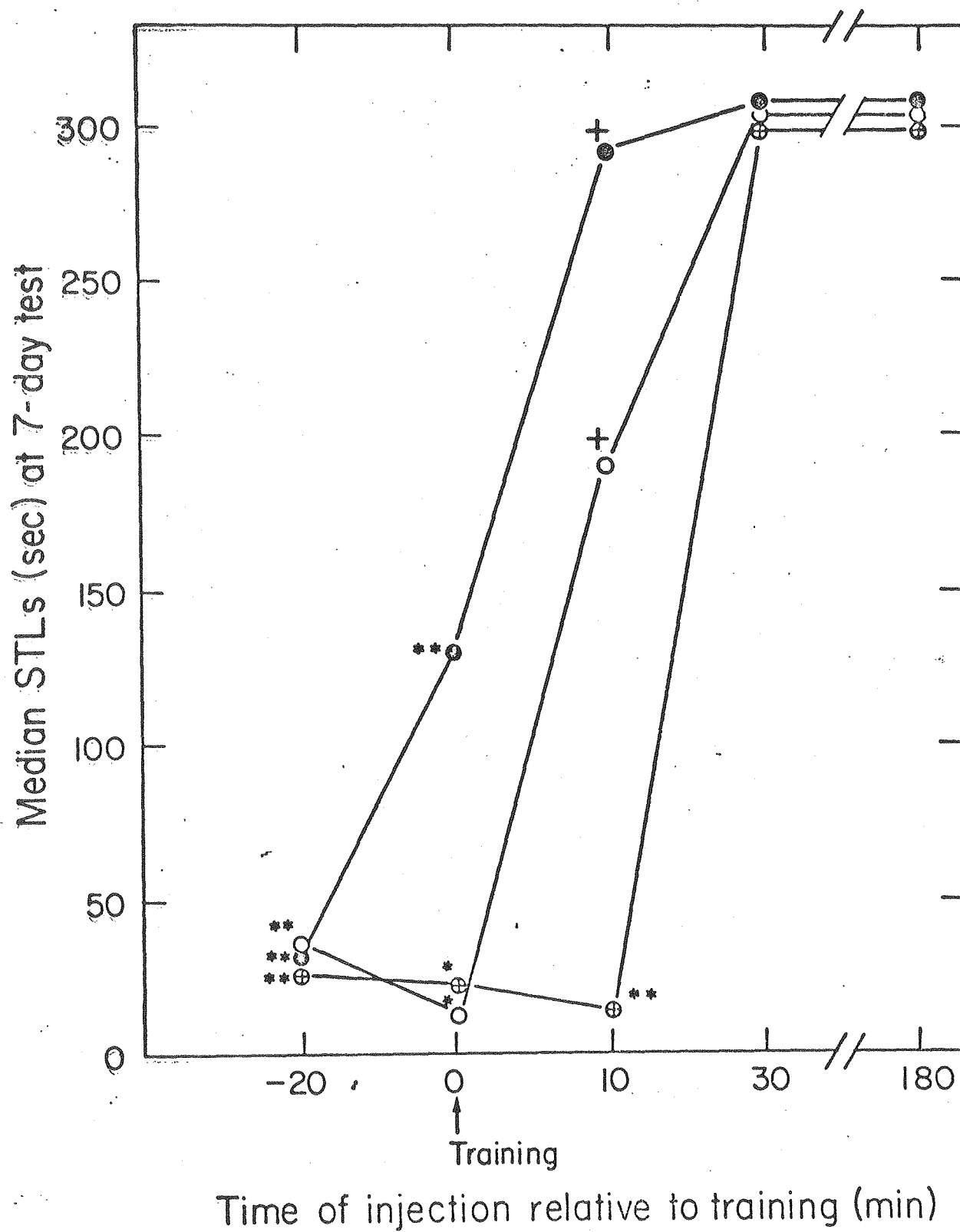
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Footnotes

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Figure 1. Median STLs of young (●—●), intermediate (○—○), and older (⊕—⊕) mice at a 7-day retention test. For each age and injection time a corresponding saline control group demonstrated a median STL of 300 sec. The N per group ranged from 15-30. * $p < 0.01$ and ** $p < 0.001$ as compared to the corresponding saline control group. ⁺ $p < 0.05$ as compared to older mice treated with ANI (120 mg/kg).

Figure 2. Percentage inhibition of protein synthesis by ANI (120 mg/kg) is presented for young (○—○), intermediate (●—●), and older mice (▲—▲). Four mice were used for each data point, and the standard errors of the mean are shown by the vertical bars.



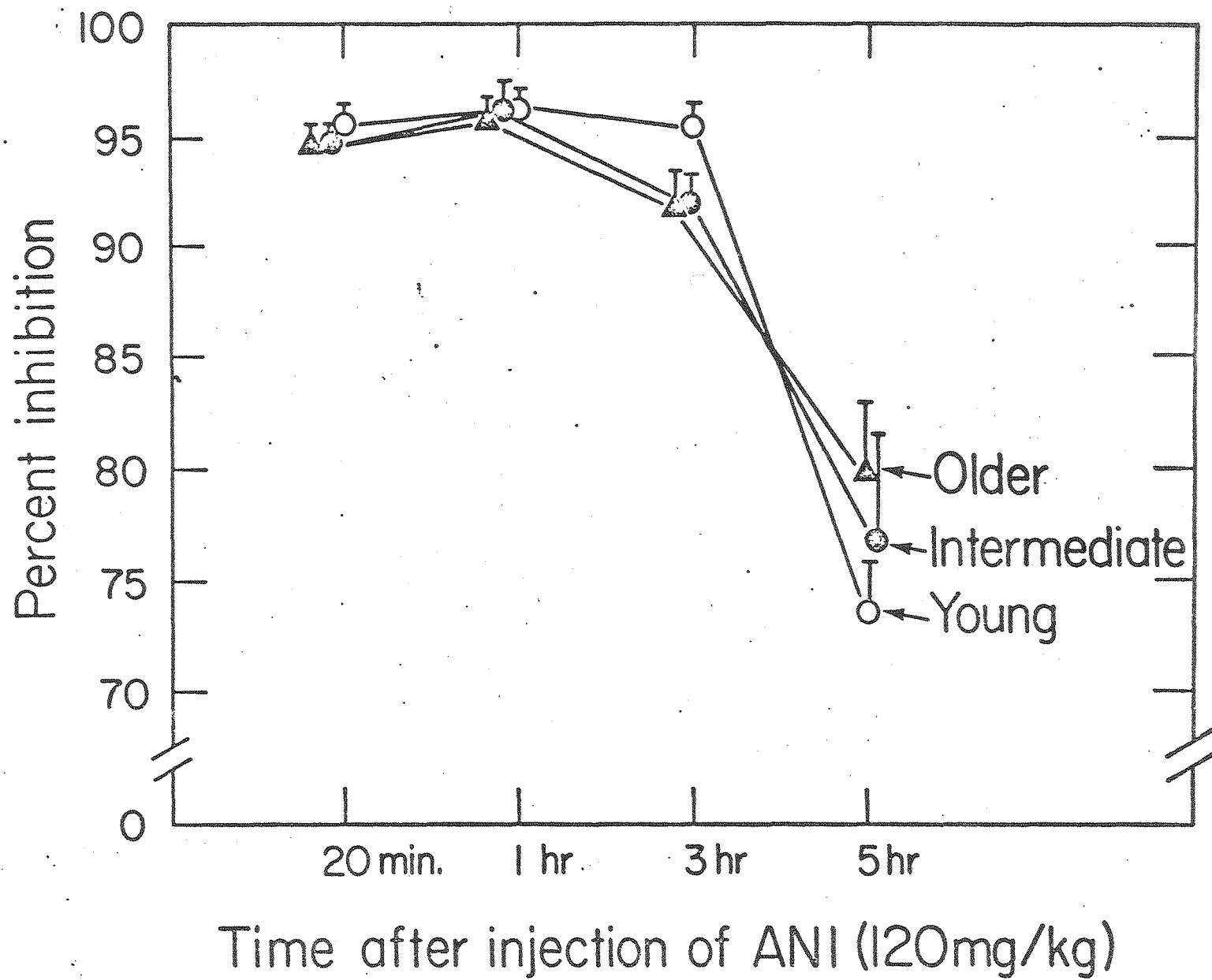


Fig. 2

XBL 809-4373

