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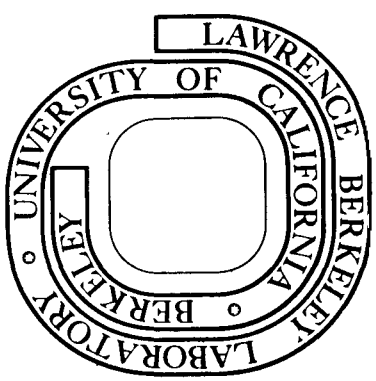

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COMPARISON OF EFFECTS ON MEMORY OF CATECHOLAMINE AND PROTEIN SYNTHESIS INHIBITORS

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ABSTRACT

Does anisomycin (ANI) produce decrements in long-term memory by effects on tyrosine levels and rate of accumulation of catecholamines rather than by its primary effects on protein synthesis? Amnesic effects of ANI were compared in six experimental paradigms with those of the catecholamine inhibitors (CAIs) diethyldithiocarbamic acid (DDC), α-methyl-p-tyrosine (AMPT), and tetrabenazine (TB). Pretraining administration of any of these drugs resulted in amnesia for passive avoidance training, but only when training was weak. With stronger training, a series of three injections of ANI (one pre- and two post-training) caused amnesia but similar series of CAI injections did not cause amnesia. Substituting one CAI injection for one of the three ANI injections did not cause amnesia, but substituting cycloheximide (another protein synthesis inhibitor) did produce amnesia. With active avoidance training, ANI caused amnesia while AMPT did not; d-amphetamine blocked the amnesic effect of ANI but potentiated the effect of AMPT. Whereas ANI lengthened the temporal gradient at which ECS produced amnesia, AMPT or DDC did not. Effects of these drugs on cerebral concentrations of tyrosine and catecholamines and on rate of accumulation of catecholamines were determined. ANI had relatively small effects, whereas the CAIs produced large reductions. When ANI and a CAI were used in combination, the pharmacological effect of the CAI predominated. We conclude that ANI and CAI have distinctly different abilities to produce amnesia and that the mechanisms of their amnesic effects differ. These experiments provide additional support for the hypothesis that protein synthesis is one component necessary for formation of long-term memories.

Many researchers have shown that protein synthesis inhibitors impair the formation of long-term memory, and it is generally accepted that inhibitors of protein synthesis such as acetoxycycloheximide, cycloheximide, and anisomycin (ANI) are effective amnestic agents in a variety of species for a variety of behavioral paradigms [1, 7, 10, 15, 24]. However, less agreement exists concerning the necessity of protein synthesis for long-term memory formation, and numerous alternative suggestions for the amnesic effects of inhibitors of protein synthesis have been advanced. One of the most persistently raised alternative explanations offered to explain the amnesic effect of inhibitors of protein synthesis is that these antibiotic agents also cause marked changes in cerebral tyrosine levels by inhibition of tyrosine hydroxylase and modify the concentrations and rates of accumulation of catecholamines [16, 17, 27]. The same research indicated that inhibition of the synthesis of catecholamine neurotransmitters might be the causal factor responsible for the amnesia induced by protein synthesis inhibitors, rather than the inhibition of protein synthesis. Quartermain has pointed out similarities of the effects of catecholamine depleting drugs and inhibitors of protein synthesis in their amnesic behavioral effects.

A related series of studies comparing amnesia induced by dopamine β-hydroxylase inhibitors and cycloheximide have also suggested that inhibition of catecholamine by protein synthesis inhibitors may also account for the resulting memory deficit [11, 32]. Quartermain et al. [31] also compared the amnesic effects of diethyldithiocarbamate (DDC), α-methyl-p-tyrosine (AMPT) and cycloheximide (CYCLO) in a left-right discrimination apparatus and found that these drugs were only effective as amnestic treatments under conditions of weak training, It should also be noted that the amnesia investigated by Quartermain et al. was transient amnesia observed 24 h after training.

Squire et al., tested the hypothesis that inhibition of brain tyrosine and the resulting increase in brain tyrosine and depletion of norepinephrine

could account for the amnesic effect of protein synthesis inhibitors [34, 35]. Neither the administration of large doses of tyrosine or AMPT produced amnesia, while inhibitors of protein synthesis did.

Our present experiments were designed to test further the hypothesis that ANI may produce a decrement in long-term memory trace formation by its side effects on cerebral tyrosine levels and on the rate of accumulation of newly synthesized catecholamines rather than by its primary effect on protein synthesis. Three catecholamine inhibitors (CAIs) which have major but different effects on tyrosine, NE, and DA were tested along with ANI in a series of experiments which allowed comparison of the correspondence of the behavioral effects. The biochemical effects of these drugs singly and in combination were also determined. The rationale of these experiments was that if ANI was acting primarily by its secondary actions on catecholamines rather than by its action as in inhibitor of protein synthesis, then one or more of the CAIs should substitute for ANI across the experimental paradigms, and in some cases the amnesic effects of a CAI might even be expected to exceed those of ANI. If, however, a correspondence of behavioral effects was not obtained over a variety of experimental designs, and if ANI was more effective than a CAI, this would provide strong support for the conclusion that protein synthesis is a necessary component of long-term memory trace formation. A second objective of this study was to compare the effects of ANI and the catecholamine inhibitors on both the conversion of tyrosine to norpinephrine and dopamine and on the concentrations of these neurotransmitters under our experimental conditions.

The results confirmed that catecholamine inhibitors can impair long-term memory formation under appropriate conditions but in a variety of experimental paradigms these inhibitors are not nearly as effective as ANI and could not be substituted for ANI. While ANI does reduce the concentrations and

accumulation of catecholamines in brain, it is not nearly as effective in this regard as the catecholamine inhibitors.

The important finding of these experiments was that CAIs did not have the same behavioral effects as the protein synthesis inhibitor, anisomycin. It is concluded that inhibitors of protein synthesis and inhibitors of catecholamine neurotransmitter synthesis cause amnesia by different mechanisms and that protein synthesis is an essential component for long-term memory-trace formation.

BEHAVIORAL EXPERIMENTS

Materials and Procedures

Animals. Swiss Webster (CD-1) male mice 60 to 80 days old at training, were obtained from Charles River Breeding Laboratories at 6 weeks of age. They were housed singly 24 hrs prior to training and until the retention test one week later.

Apparatus and Training Procedures

Step-through passive avoidance. The procedure for training and testing mice for the one-trial, step-through passive avoidance task has been described previously ([21]. In brief, the one-trial, step-through apparatus consisted of a black start compartment joined to a white shock compartment by a partition containing a mousehole through which the subjects could enter the white compartment. In the white compartment, footshock was given until the mouse returned to the black compartment. The strength of learning was determined by the shock-intensity. For a given shock intensity, only subjects both entering in one to three seconds and escaping in one to three seconds were used. To test retention, the mice were again placed into the black compartment and the time required for the subject to enter the white compartment was taken as a measure of retention. A latency-to-enter the white shock compartment on the test day of 20 sec or less was defined as amnesia. Most trained non-amnesic mice did not enter the white compartment within the three-minute test period. Percentage amnesia is defined as the percentage of mice having an entry time of less than 20 sec. Training and testing were done between the hours of 7:30 AM and 2 PM.

T-Maze active avoidance. The T-maze apparatus and training have been previously described [20]. The training apparatus consisted of a black Plexiglas start alley with a start box at one end and two goal boxes at

the other fitted with clear plastic liners used to remove the mouse from the box. The bottoms of these liners went below the shock grid which ran throughout the entire maze. The start box was separated from the rest of the start alley by a black Plexiglas guillotine door which prevented the animal from moving down the start alley until the trial started. A door-bell type buzzer served as the conditioned stimulus. The intertrial interval was about 45 sec.

Training consisted of placing the mouse in the start box, then raising the guillotine door and simultaneously sounding the buzzer. Mice not moving to the correct goal box within 5 sec received footshock until they did so. The side preference was determined on the first training trial by forcing all mice to go to the side opposite to their first response. On subsequent trials, the correct side was the non-preferred side for each mouse. At the end of each trial, the mouse was removed to its home cage by carefully removing the liner and placing it into the mouse cage. As training proceeded, a mouse could make one of two types of responses: (a) a response latency longer than 5 sec was an escape, (b) a response latency of 5 sec or less was an avoidance. The retention test was given one week after training and consisted of retraining the mouse to a criterion of one conditioned response, that is an avoidance response to the correct side of the T-maze. Percentage amnesia is defined as the percentage of mice requiring more than 3 trials to make an avoidance response.

Drugs. All drugs and control saline injections were administered subcutaneously without any anesthetic. Anisomycin (ANI) (Pfizer Diagnostics, Clifton, NJ) was administered at 20 mg/kg, diethyldithiocarbamic acid (DDC) (Sigma Chemical Co., St. Louis, MO) at 250 mg/kg, α-methyl-p-tyrosine (AMPT) (ICN-Nutritional Biochemical Co., Cleveland, OH) at 160 mg/kg, tetrabenazine

(TB) (Hoffman-LaRoche, Nutley, NJ) at 10 mg/kg, and d-amphetamine sulfate at 0.2 mg/kg. DDC, AMPT, and TB were administered at the highest possible doses that did not impair the subject's motor ability. All drugs were prepared in physiological saline and the pH was adjusted in each case to between 6 and 7, except for TB which was adjusted to a pH of 5.0 to 5.5.

Experiment 1. Effect of Training Footshock Intensity on Effectiveness of Amnestic Agents.

The purpose of this experiment was to compare the effectiveness of pretraining injections of catecholamine inhibitors (CAIs) with that of ANI, a protein synthesis inhibitor, as a function of strength of training as determined by foot shock intensity. Subjects were trained on the one-trial passive avoidance task as described above at the following footshock intensities: 0.32, 0.34, and 0.36 mA. The three CAIs (DDC, AMPT, and TB) and saline were administered 60 min and ANI 15 min prior to training. The reason for the difference in time of administration was to allow sufficient time for the slower acting CAI to exert an effect on neurotransmitter concentrations and synthesis. ANI achieves 85 to 90% inhibition of whole brain protein synthesis within less than 5 min [9, and in preparation]. An N of 20 was obtained for each group. Retention was tested one week after training.

Results

At the lowest footshock intensity (0.32 mA) used for training, the CAIs and ANI led to a significantly greater percentage of amnesic mice than found after a saline injection (Table 1). As the footshock intensity increased, the effectiveness of the CAIs (DDC, AMPT, and TB) and a single injection of ANI as amnestic agents decreased approximately in parallel. At the highest footshock intensity (0.36 mA) none of the CAIs nor ANI yielded retention scores differing significantly from saline. This confirmed our previous result that as the strength of training increased, the effectiveness

of an amnestic agent decreased [21]. However, this experiment did not provide evidence that the effective mode of action of these drugs differed.

Experiment 2. Comparison of Effects of Administration of Multiple Doses of CAI or ANI on Retention.

Previous research [22] has shown that longer duration of inhibition of protein synthesis could overcome an increase in training strength and re-establish amnesia. The purpose of this experiment was to compare the effectiveness in producing amnesia of a series of three injections of each of the CAIs with a series of three injections of ANI. The subjects were trained on the one-trial passive avoidance task as in Exp. 1.

Results

Three successive injections of ANI (ANI^3) yielded 90% amnesic subjects which can be compared with 10% amnesic subjects for three successive injections of saline. ANI^3 also produced a significantly greater percentage of subjects classed as amnesic than three successive doses of DDC, AMPT, or TB (Table 1, lower half). None of the CAIs resulted in a significantly greater percentage of amnesic subjects than the saline controls. Thus, at this higher level of passive avoidance training, multiple injections of a CAI did not have the same effect on retention as multiple injections of ANI, a protein synthesis inhibitor.

Experiment 3. Amnesic Effect and Time of Drug Administration.

Since the amnesic effect of ANI is hypothesized to be related to the duration of inhibition of protein synthesis immediately after training, then one would expect that the amnesic effect would decrease the longer before training the ANI is administered since this would shorten the duration of protein synthesis inhibition after training. The purpose of the experiment was to determine if CAIs and ANI showed the same type of time dependent effect on retention. The subjects and training were as in Experiment 1. The footshock was set at 0.34 mA so that increases or decreases in amnesia

could be observed at injection times other than 1 hr. Other conditions were as in Experiment 1. ANI, DDC, or AMPT were administered to separate groups of animals either 15 min, 1 or 2 hr prior to training. The N/group is given in Table 2.

Results

The time of the injection per se had no effect on retention as the saline controls showed 20-25% forgetting across the injection times. ANI yielded an amnesic gradient that showed that it was most effective as an amnestic agent the closer to training that it was administered [ANI (-2 hr) vs ANI (-15 min); $\chi^2 = 5.33$ $P < .05$]; in fact, when administered 2 hr prior to training ANI had no significant amnesic effect (Table 2). DDC yielded a nearly constant amnesic effect over the time period. AMPT was clearly more effective as an amnestic agent when administered 2 hr prior to training than 15 min prior to training ($\chi^2 = 4.82$, $P < .05$).

Experiment 4. Effect of Substitution of a CAI for ANI.

If the mode of action of a protein synthesis inhibitor as an amnestic agent is a result of its inhibition of CA synthesis, then one should be able to replace one of the series of three ANI injections with an injection of a CAI and still obtain a high percentage of amnesic subjects. To test this hypothesis, subjects were trained as in Experiment 2 on the one-trial passive avoidance task. ANI was administered 15 min prior to training to all groups except the saline controls. The second injection was either ANI or CYCLO (1-3/4 hr after training) or TB, AMPT, DDC or saline (administered 3/4 hr after training). All groups except the saline control (SALINE + SALINE + SALINE) received an injection of ANI at 3-3/4 hr after training. The N group was 20; retention was tested one week after training and drug treatment.

In the groups receiving either TB, AMPT, DDC or saline, recovery of protein

synthesis occurred because the second ANI injection was omitted [19]. The CAIs were administered earlier than the protein synthesis inhibitors so that they would have time to inhibit CA synthesis prior to the resumption of protein synthesis. If inhibition of CA synthesis is critical to ANI-induced amnesia, then substituting TB, AMPT, or DDC for the second ANI injection should result in as high a percent amnesia as ANI + ANI + ANI or ANI + CYCLO + ANI. If, on the other hand, inhibition of protein synthesis is a principle mechanism of ANI or CYCLO-induced amnesia, then ANI + (TB, DDC, or AMPT) + ANI should yield retention scores comparable to ANI + SALINE + ANI.

Results

ANI + ANI + ANI and ANI + CYCLO + ANI both differ significantly from ANI + SALINE + ANI ($P < .001$ in both cases, χ^2 Test). Substituting DDC, AMPT, or TB for the second ANI injection did not result in a high percentage of amnesic subject (Fig. 1). In fact, none of the ANI + CAI + ANI groups differ significantly from ANI + SALINE + ANI. Thus, while cycloheximide, an inhibitor of protein synthesis, could be substituted for ANI and result in a high percentage of amnesic subjects, the catecholamine inhibitors, DDC, AMPT, or TB, could not.

Experiment 5. Anti-amnesic Effect of d-Amphetamine.

d-Amphetamine blocks the amnesic effect of protein synthesis inhibitors [8, 23, 25, 26, 28]. The purpose of this experiment was to determine if d-amphetamine had the same anti-amnesic effect against AMPT-induced amnesia as against ANI-induced amnesia. AMPT was chosen because it blocks dopamine and norepinephrine synthesis and d-amphetamine is believed to act primarily on dopaminergic synapses [12, 30, 33].

Subjects were trained on the T-maze active avoidance task with a foot-shock intensity of 0.33 mA. ANI and AMPT were administered under different injection schedules because of the differing times required to exert known

pharmacological effects. AMPT (or saline) was administered subcutaneously one hr prior to training at a dose of 160 mg/kg; d-amphetamine or saline was administered intraperitoneally immediately after training. The three successive injections of ANI required to cause amnesia along with the corresponding saline control injections were given 15 min prior to training, and 1-3/4 hr and 3-3/4 hr after training.

Results

AMPT was not effective at blocking retention in this active avoidance task, while ANI yielded significant amnesia (Table 3). d-Amphetamine significantly reduced the amnesic effect of the three successive injections of ANI (ANI^3 + SALINE = 80% vs. ANI^3 + d-Amphetamine = 20% amnesia; $P < .01$, χ^2 Test). Surprisingly, d-amphetamine did not reduce the percentage amnesia in the AMPT group but increased the amnesic effect (AMPT + SALINE = 25% amnesia compared with AMPT + d-Amphetamine = 90% amnesia; $P < .01$, χ^2 Test).

Experiment 6. Interaction of Inhibitors with ECS.

Inhibitors of protein synthesis (cycloheximide or ANI) can greatly lengthen the gradient for ECS retrograde amnesia [2, 3, 18]. In these experiments, ECS given alone was found to be an effective amnestic treatment up to 30 min but not 60 min after training. The administration of cycloheximide prior to training extended the length of the ECS gradient to at least 3 hr. Using 1, 2 or 3 injections of ANI, Flood et al. [18] showed that the length of the ECS gradient was directly related to the duration of protein synthesis inhibition such that ECS was an effective agent up to 3 hr after a single ANI injection, 5 hr after two injections and up to 7 hr after three successive injections of ANI.

The purpose of this experiment was to determine if AMPT or DDC would similarly extend the length of the ECS retrograde amnesic gradient. Subjects were trained on passive avoidance following the general procedures described

above. The footshock intensity was 0.40 mA in order to provide strong training, and the latency-to-enter and escape was within the range of 1.0 to 3.0 secs. ANI was administered 15 min prior to training and 1-3/4 hr after training. DDC, AMPT, or saline were administered 1 hr prior to training and 1-3/4 hr after training at the doses and in the manner described above. ECS was administered transcorneally 3 hr after training at 8 mA for 0.2 sec at 60 Hz; only a few subjects were discarded for failing to show both tonic and clonic convulsions and several subjects died as a result of the ECS administration. No respiratory assistance was given to the mice receiving ECS. Mice given pseudo-electroconvulsive shock (P-ECS) were handled in the same manner as the ECS mice except that no current was delivered.

Results

The ANI + ANI/P-ECS groups were not amnesic; therefore, any amnesic effect of ANI and ECS treatments could not be due to administration of the protein synthesis inhibitor alone (Table 4). SALINE + SALINE/ECS did not result in amnesia showing that ECS given 3 hr after training does not cause amnesia. But, when ANI and ESC are combined, 92% of the subjects were classed as amnesic (ANI + ANI/ECS vs SALINE + SALINE/ECS, $\chi^2 = 19.74$ $P < .001$). Neither the DDC + DDC/ECS nor the AMPT + AMPT/ECS treatments significantly lengthened the gradient of ECS retrograde amnesia. However, AMPT + AMPT/ECS had a Chi-Square value that was close to the .05 level of significance. As expected from the high level of the footshock, none of the drug and P-ECS conditions showed appreciable forgetting.

BIOCHEMICAL EXPERIMENTS

The purpose of the biochemical experiments was to determine the relative effects of ANI and the CAI's on the concentration and accumulation of newly synthesized catecholamines under our experimental conditions. The conclusion from the behavioral experiments that inhibition of protein synthesis is more effective in producing amnesia than reduction of catecholamine levels and/or turnover might not be tenable if either ANI by itself changed the CA concentration or if the combination of ANI and CAI impaired the action of a CAI.

General Description of Procedure

The Swiss-Webster mice, ANI, and the CAIs were obtained from the suppliers listed in the Behavioral Section. Solutions were freshly prepared at the appropriate concentrations. L-[2,6-^{3}H]-tyrosine (49 Ci/mM) was obtained from Amersham. Sources of the standards were: tyrosine, Cal Biochem; norepinephrine and dopamine, Regis.; and quinine sulfate used for standardization of the spectrofluorometer, Aldrich. The other chemicals were reagent grade and were obtained from standard commercial suppliers.

General Procedure for the Determination of Concentrations of Tyrosine, Norepinephrine, and Dopamine.

Numerous procedures have been published for the determination of catecholamines; many of these procedures are variants of basic procedures published by Anton and Sayre [4, 5]. The procedures used are outlined below and were adopted as best suiting our needs after careful evaluation of alternate procedures.

Drug administration and sacrifice. On Day 1, mice were given as appropriate, saline, ANI, and/or the CAI's by subcutaneous injection To determine turnover of DA and NE, 15 µc of [^{3}H]-tyrosine was injected subcutaneously 15/$\overset{\text{min}}{}$prior to sacrifice. The times from drug administration

to sacrifice are shown in Tables 5 thru 7. When ANI plus a CAI were administered, the CAI was administered 1 hr after ANI, and the mouse was sacrificed 1 hr later. Animals were killed by cervical dislocation, brains (including cerebellum and medulla) were rapidly excised, weighed, and frozen on dry ice. The brain samples were stored in a deep freeze overnight on dry ice.

On Day 2 the brain sections were homogenized and tyrosine was separated from NE and DA on microcolumns following the procedures described by Barchas and co workers [6, 29]. Typically two brains were combined and homogenized in a Potter Teflon-glass homogenizer for 1 min in 3.5 ml of 0.4 N $HClO_4$ plus 0.1 ml of 10% EDTA. The homogenate was centrifuged at 30,000 g for 20 min and the precipitate washed with 1.5 ml of 0.4 N $HClO_4$ and recent rifuged. The wash was combined with the first supernatant, and the combined supernatants were adjusted to pH 6.0 $\pm$ 0.15 by the careful addition of 2N KOH. Since the catecholamines are destroyed rapidly under alkaline conditions, the neutralization was carefully controlled using an autotitrator. The precipitated $KClO_4$ was removed by centrifugation, and the volume of the supernatant was adjusted so that 1 ml was equivalent to 150 $\pm$ 10 mg of initial brain weight.

Microcolumn separation of catecholamines. Microcolumns containing Fisher Rexyn 102 (instead of Bio-Rex 70) were prepared following the description of Holman et al [29]. Immediately prior to use, a column was washed with 1.5 ml of 0.1% EDTA, and then 2.0 ml of the neutralized extract from the brain was placed on the column. Each brain homogenate was assayed in duplicate. Tyrosine was eluted with 1.5 ml of 0.02 M sodium phosphate buffer, pH 6.5 containing 0.2% EDTA, followed by three 1-ml washes of 0.1% EDTA. At least

99% of added tyrosine is recovered in this fraction. Total tyrosine and radioactive tyrosine were determined in the combined eluate and wash. DA and NE were then eluted from the column by 2 ml of 0.5 N acetic acid. Pilot studies indicated that 85 to 92% of added NE and DM were recovered in this fraction. Gentle pressure was applied with N_2 gas to remove all of the liquid from the column and to provide an oxygen-free atmosphere for the CA. The extracts were stored in the dark at -5° under N_2 until analyzed. The radioactive tyrosine converted to DA and NE was determined using .5 ml aliquots of acetic acid eluted material. Conversion rate was based upon the radioactivity in the catecholamines and the final specific activity of the tyrosine. The actual conversion rate is perhaps as much as two-fold higher.

Catecholamine assay. To minimize oxidation, the DA assays were normally completed the day and evening following the column elution. During assay, samples were stored in an icebucket with a lid to prevent photooxidation, and N_2 gas was added to the top of the tube after dopamine assay to minimize oxidation of Ne.

The assays developed by Anton and Sayre [5] were used for DA and NE since, of several methods we tested, they were the most reproducible and convenient to use for a large number of samples. Fluorescence of the dihydroxyindole derivative from DA was measured with excitation at 316 nm and emission at 370 nm in a Perkin-Elmer Fluorescence spectrophotometer Model MPF-2A. The sample blank was prepared by adding the sample aliquot last to the mixture of reagents; the internal standard was prepared by the addition of 10-20 ng DA to the sample aliquot.

Following the Anton and Sayre procedure [5], NE was converted to 3,5,6-trihydroxyindole with potassium ferricyamide and alkaline ascorbate. The emission fluorescence at 519 nm of the indole due to excitation at 409 nm was determined.

The tissue blank was obtained by addition of the sample aliquot last to the mixture of reagents; the internal standard was prepared by addition of 2.5 ng of NE to the sample aliquot. Quinine sulfate (10^{-6}M) was used to standardize the fluorescence spectrophotometer.

Tyrosine assay. Tyrosine is more stable than the catecholamines, and its assay was done on the day subsequent to the determination of the catecholamines. The assay was based upon the formation of a phenoxazinone derivative from tyrosine and nitrosonaphthol in the presence of nitrate-nitrite catalyst as described by Udenfrient [36]. In the first series of experiments (Table 5), the column eluate was made 6% in trichloroacetic acid. Fluorescence of phenoxazinone in the aqueous phase was determined by emission at 570 nm upon excitation at 460 nm. Fluorescence of appropriate internal standards and blanks were also determined.

In a second series of experiments tyrosine was determined by a more direct procedure with minor modification of the above assay. In this procedure, a mouse brain was homogenized in approximately 5 ml of 0.4 N $HClO_4$ and 0.1 ml of 10% EDTA. The solution was centrifuged at 9,000 g for 10-15 min; the pellet was resuspended and washed in 2 ml of 0.4 N $HClO_4$ and recentrifuged. The combined supernatants are recentrifuged at 30,000 g for 15 min to eliminate any turbidity which would interfere with the fluorescence measurement. The supernatant was adjusted with 0.4 N $HClO_4$ to be equivalent to 50 mg of tissue/ml, and was stored in the refrigerator until assayed the next day. The assay for tyrosine was done as described above except 0.5 ml of water was used instead of 0.5 ml of 12% trichloroacetic acid which depressed the sensitivity. The tyrosine standard was prepared in H_2O, and 0.5 ml used as the internal standard contained 0.5 µg of tyrosine.

Inhibition of Protein Synthesis

The inhibition of protein synthesis by a CAI, ANI, and combination of ANI plus a CAI was determined using L-[U-^{14}C]-valine as the protein precursor by our standard procedure [13, 18].

RESULTS

Experiment 1. Effects of ANI on Tyrosine and Catecholamines.

An extensive series of experiments was performed to determine the effects of ANI on brain concentrations of tyrosine and CA and on conversion of tyrosine to CAs using several drug dosages and at several times after ANI administration. The results indicate that irrespective of dose or time after administration of ANI, the major effect is to cause a large increase of 40 to 70% in cerebral tyrosine concentration from the normal value of approximately 19 µg/g brain (Table 5). However, no significant change in either the DA or NE content of brain was found as a result of ANI administration. An apparent decrease in the conversion of tyrosine to CAs was noted. However, this was probably an artifact due to the increase in the precursor pool of tyrosine. At long periods after administration of a series of three doses of ANI a significant decrease in conversion of tyrosine to CAs may have occurred.

Experiment 2. Effects of CAIs on Tyrosine and Catecholamines.

A second series of experiments investigated the effects of DDC, TB, and AMPT on tyrosine and catecholamines (Table 6). At the doses used in the behavioral experiments DDC caused a decrease in NE concentration and also in the conversion of tyrosine to CAs. At a dose four times that used in the behavioral experiments, the concentration of tyrosine was increased. TB drastically lowered the concentration of both DA and NE, but had only a marginal effect on the conversion to tyrosine into CAs. AMPT caused

moderate reductions in DA and NE concentrations at 1 hr, and somewhat larger reductions at 2 hr after administration. The effect on tyrosine concentration was not determined due to AMPT interference in the tyrosine assay.

Experiment 3. Effects of Combined Administration of ANI and a CAI.

A third series of experiments investigated the effects of administration of ANI followed by a CAI. Not unexpectedly, in each case the action of the CAI prevailed and the resulting levels of the CA were comparable to those obtained with the CAI alone (Table 6).

Experiment 4. Effects of CAIs on Protein Synthesis.

The effects of a series of three injections of CAIs at two hr intervals on protein synthesis was compared to an equivalent series of ANI injections. In addition, the combined effect of ANI plus a CAI was also determined. Of the CAIs tested, DDC was the most effective inhibitor of protein synthesis, but the inhibition at 1 hr after the last of three injections was less than one-half of that obtained with ANI under similar conditions (Table 7). A CAI in combination with ANI caused only a slight increase in inhibition of protein synthesis over that caused by ANI alone.

DISCUSSION

The present experiments were designed to test further the hypothesis that ANI may produce a decrement in long-term memory trace formation by its side effects on cerebral tyrosine levels and on the rate of accumulation of newly synthesized catecholamines rather than by its primary effect on protein synthesis. Three CAIs which have major but differing effects on tyrosine, NE and DA were tested along with ANI in a series of experiments that allowed comparison of the biochemical and behavioral effects.

Experiment 1 was the single experiment in which the CAIs mimicked the effects of ANI. However, these drugs were only effective at the low footshock intensity. The results of this experiment are in accordance with those

of Quartermain [31] discussed above. Experiment 2 showed that under conditions of stronger training for a passive avoidance task, the CAI did not cause amnesia even when a series of three injections was given, whereas ANI did. In Experiment 3, the amnesic effect of ANI and the CAIs did not show the same temporal relationship between time of pretraining injection and percent forgetting. ANI was more effective when given 15 min prior to training than at 1 or 2 hr prior. DDC was as effective at 15 min as at 1 or 2 hr prior to training, and AMPT was decidedly more effective when administered 2 hr prior to training than 15 min prior to training. In Experiment 4, it was shown that if the second of a series of three ANI injections was changed to CYCLO, another protein synthesis inhibitor, a high percentage of amnesic subjects was again obtained. However, when DDC, AMPT, or TB was substituted for the second ANI injection, no significant amnesic effect was observed. In fact, the CAIs had no greater effect on retention than a saline injection administered at the same time. In Experiment 5, d-amphetamine blocked the amnesic effect of ANI, but significantly potentiated the amnesic effect of AMPT. This again suggests different mechanisms of action involved in producing amnesia. d-Amphetamine is thought to act by blocking the release or uptake of dopamine [12, 30, 33]. With AMPT blocking the synthesis of additional dopamine and d-amphetamine preventing release or uptake of dopamine, a more rapid reduction of transmitter with its receptor must result when the drugs are employed together. Since d-amphetamine improved retention in both non-inhibited control and ANI-injected mice, these results further suggest that ANI did not block dopamine synthesis to the extent that AMPT did. This suggestion is in line with the biochemical determinations which showed that ANI had little or no effect on DA concentration in the brain whereas AMPT caused a marked reduction in DA concentration.

Our biochemical experiments confirm the previous reports of Flexner et al. [16] and Squire et al. [35] that ANI caused a marked elevation in cerebral concentrations of tyrosine. The magnitude of this elevation (approximately 50%) was not markedly dependent upon either the dose of ANI or the duration of inhibition. We did not find a significant change in the concentrations of DA or NE after ANI administration. These results were also in agreement with those of Flexner and Goodman [16]. But in contrast to their report, we failed to find a major change in the rate of conversion of [2,6H^3]tyrosine to the combined DA and NE fraction. Squire et al [35] have reported that the inhibition of tyrosine hydroxylase is only about 8% after the administration of 150 mg/kg of ANI. In our view, it is probable that the increase in tyrosine concentration is due to the inhibition of protein synthesis rather than to the inhibition of the hydroxylase activity.

DDC at the doses used in the behavioral experiments caused a 40% reduction in NE concentration, AMPT caused only a modest reduction in DA and NE concentrations, while TB caused drastic reduction in both DA and NE. When these CAIs were combined with ANI, the pharmacological action of the CAIs prevailed.

The main conclusion from our experiments is that inhibitors of protein synthesis and inhibitors of CA neurotransmitter synthesis cause amnesia by different mechanisms. The biochemical studies showed that in general ANI did not significantly reduce DA or NE levels in the brain and that CAI's did not inhibit protein synthesis to any great extent. This suggests that these substances cause amnesia in different ways. The behavioral results showed that in general one could not use protein synthesis inhibitors and CAI's interchangeably and obtain the same amnesic effect. This also suggests different mechanisms of action. If the mechanism of action of these two types of inhibitors was the same, then one would expect to find a far greater similarity in their behavioral and biochemical effects.

We do not wish to suggest that neurotransmitters are not important

for long-term memory formation, since certainly the transmission of nerve impulses must play a role. But our results demonstrate that blocking the metabolism of neurotransmitters with low doses of DDC, TB, or AMPT administered prior to or shortly after training is not effective in causing amnesia.* Since administering ANI at these times is an effective amnestic treatment, it is clear that ANI's principal amnestic mechanism does not involve inhibition of the catecholamine system. All of the available evidence is therefore still consistent with the theory that synthesis of brain proteins is required for long-term memory trace formation, and that ANI, and most likely cycloheximide and acetoxycycloheximide, are effective due to their action as inhibitor of protein synthesis [10].

Footnote:

*Amnesia from post-trial administration of high doses (900 to 2000 mg/kg) of DDC has been reported [14, 28, 37[, but these high doses may produce many secondary effects in neurotransmitter metabolism.

TABLE 1

AMNESIC EFFECT OF CA AND PROTEIN SYNTHESIS INHIBITORS ACROSS TRAINING FOOTSHOCK INTENSITIES

Drug Group	Footshock Intensity (mA) 0.32	0.34	0.36
		%Forgetting*	
	Experiment 1		
SALINE	20	20	20
DDC	75	25	20
AMPT	85	50	20
TB	70	45	25
ANI	75	65	20
	Experiment 2		
SALINE[3]			10
DDC[3]			10
AMPT[3]			30
TB[3]			10
ANI[3]			95

*N = 20/group

TABLE 2

PERCENT AMNESIA AS A FUNCTION OF THE TIME

OF PRE-TRAINING DRUG ADMINISTRATION

	N	Time -2 hr	N	Time -1 hr	N	Time -15 min
Saline	17	24	20	20	20	25
ANI	18	33	14	64	15	73
DDC	14	64	12	58	14	57
AMPT	15	67	13	54	15	27

TABLE 3

EFFECT OF D-AMPHETAMINE WITH AMPT OR ANI

Drug Treatment	N	% Amnesia
CA Synthesis Inhibitor		
Saline(Saline)	20	10
AMPT(Saline)	20	25
Saline(d-Amphetamine)	20	0
AMPT(d-Amphetamine	20	90
Protein Synthesis Inhibitor		
Saline(Saline)+Saline+Saline	10	20
ANI(Saline)+ANI+ANI	10	80
ANI(d-Amphetamine)+ANI+ANI	10	20
Saline(d-Amphetamine)+Saline+Saline	10	0

TABLE 4

EFFECT OF CATECHOLAMINE AND PROTEIN SYNTHESIS INHIBITORS AND ELECTROCONVULSIVE SHOCK ON RETENTION

Treatment Group	N	% Amnesia
ANI+ANI/ECS	12	92
ANI+ANI/P-ECS	14	14
DDC+DDC/ECS	14	14
DDC+DDC/P-ECS	14	7
AMPT+AMPT/ECS	14	29
AMPT+AMPT/P-ECS	15	0
SALINE+SALINE/ECS	15	7
SALINE+SALINE/P-ECS	13	15

Table 5

EFFECTS OF ANI ON TYROSINE AND CATECHOLAMINES

Injection Schedule	Dose mg/kg	N	Time of Sacrifice* (hr)	Percent of Control** Tyrosine	DA	NE	Conversion
ANI	20	7	1/4 to 1-1/2	142	110±17	97±11	90
ANI	40	3	1	155	105	98	80
ANI	70	3	1-1/2	146	112	98	--
ANI+ANI+ANI	20+	9	1	---	92±10	83±6	46±17
ANI	20	5	1	164±20***	---	---	---
ANI+ANI	20+	5	1	169±17***	---	---	---
ANI+ANI+ANI	20+	5	1	132±6***	---	---	---

*Time of sacrifice is given as time after the last injection of ANI. When a series of ANI was administered, the injections were at two-hour intervals.

**The control values were: tyrosine, 19.1±2 µg/g wet weight brain; dopamine, 1.16±0.2 µg/g; norepinephrine, 0.29±.02 µg/g, and the conversion rate of tyrosine to CA was calculated to be 2.94 nM/g/h or 0.530 µg/g/h without correction for recovery. This conversion rate is calculated on the basis of final specific activity of tyrosine and is a low estimate.

***Tyrosine was determined by the direct assay procedure.

+Dose per injection.

TABLE 6

EFFECTS OF CAIs AND ANI ON TYROSINE AND CATECHOLAMINES

Drug	Dose mg/kg	N	Time of Sacrifice (hr)	Percent of Control*			
				Tyrosine	DA	NE	Conversion to CA
DDC	250	8	1	115±21	97±6	60±8	71±37
	1000	3	1	180± 6	80±3	48±2	82±13
TB	10	2	1	93	11	30	79
AMPT	160	8	1	---	77±21	84±11	
	160	2	2	---	40	68	
ANI*(20 mg/kg) combined with:							
DDC	250	2	1	195±28**	90	49	
TB	10	2	1	140± 7**	10	40	
AMPT	160	2	1	---	66	65	

*The CAIs were administered 1 hr after the ANI and the mice were sacrificed 1 hr later or a total of 2 hr after the ANI administration.

**Tyrosine was determined by the direct method and the N was 5 for each group. The result is given as the mean ± the standard deviation.

FIGURE LEGENDS

Fig. 1. The effect of substituting cycloheximide or catecholamine inhibitors for ANI on memory for a passive avoidance task. A high footshock intensity (0.36 mA) was used. Percent amnesia is indicated by the shaded area.

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