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Neurochemical and Behavioral Effects of Catecholamine and Protein Synthesis Inhibitors in Mice

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FLOOD, J F, G E SMITH, E L BENNETT, M H ALBERTI, A E ORME AND M E JARVIK *Neurochemical and behavioral effects of catecholamine and protein synthesis inhibitors in mice* PHARMACOL BIOCHEM BEHAV 24(3) 631–645, 1986 —A series of biochemical and behavioral experiments tested the hypothesis that anisomycin (ANI), a protein synthesis inhibitor, produced decrements in long-term memory by raising free tyrosine levels and by the accumulation of catecholamines (CAs) rather than by its primary effect on protein synthesis We compared the effects of ANI and three catecholamine synthesis inhibitors (CAIs)—diethyldithiocarbamic acid, alpha-methyl-p-tyrosine, and tetrabenazine—on cerebral concentrations of tyrosine and CAs and on the rate of accumulation of CAs ANI had a relatively small effect, whereas the CAIs resulted in large reductions When ANI and a CAI were used in combination, effects on CA levels were determined mainly by the CAI The amnestic effects of ANI and the CAIs were also compared across seven experimental paradigms Pretraining administration of any of the four drugs could result in amnesia for passive avoidance training, but only when training was weak With an increase in training strength, a series of three injections of ANI (one pre- and two post-training) caused amnesia, but a similar series of CAI injections did not Substituting one CAI injection for the second of three successive ANI injections did not cause amnesia, but substituting cycloheximide, another protein synthesis inhibitor, resulted in amnesia With an active avoidance test, ANI caused amnesia while AMPT did not, d-amphetamine blocked the amnestic effect of ANI but caused amnesia in AMPT injected mice Whereas ANI lengthened the temporal gradient over which electroconvulsive shock produced amnesia, AMPT or DDC did not DDC caused only transient amnesia for passive avoidance training, while the amnestic effect of ANI remained constant at 24-hr and 1-week retention tests We conclude that ANI and CAIs have distinctly different abilities to produce amnesia These experiments provide additional support for the hypothesis that protein synthesis is required for formation of long-term memory

Active avoidance Alpha-methyl-p-tyrosine Anisomycin Brain tyrosine Cycloheximide Catecholamines Catecholamine synthesis rate Diethyldithiocarbamic acid Memory retention Protein synthesis inhibition Passive avoidance Tetrabenazine

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RESEARCH using a variety of species and tasks has shown that protein synthesis inhibitors (acetoxycycloheximide, cycloheximide, and anisomycin) are effective amnestic agents [1, 2, 6–10, 15, 16, 18, 27, 32, 46] However, in spite of the large number of reports demonstrating impaired retention in animals treated with a protein synthesis inhibitor, controversy exists as to whether the principal action (protein synthesis inhibition) or a secondary action of the inhibitor is responsible for the observed amnesia [17] One alternative hypothesis suggests that the antibiotics modify the concentrations of catecholamines and the rates of catecholamine synthesis and thereby affect memory formation [20, 33, 34, 47] The catecholamine hypothesis has been explicitly addressed in a number of previous reports [29, 49, 50] as well as in several reviews [15, 16, 47]

In behavioral experiments using reversal training, Quartermain and co-workers found that any of the following agents induced transient amnesia which was alleviated by pre-testing injections of drugs that elevated catecholamine levels [12, 41–44] diethyldithiocarbamate (DDC) a blocker of dopamine (DA) conversion to norepinephrine (NE), alpha-methyl-p-tyrosine (AMPT), a competitor for tyrosine as a substrate for DA synthesis, and cycloheximide (CYCLO), an inhibitor of brain protein synthesis The results suggest the involvement of biogenic amines in CYCLO-induced amnesia

The purpose of our experiments was to test further the hypothesis that anisomycin (ANI), an inhibitor of brain protein synthesis, causes amnesia by its side effects on the rate of accumulation of newly synthesized catecholamines (CAs) rather than by its primary effect on protein synthesis

The main purpose of the biochemical experiments was to determine the relative effects of ANI and the CAIs on the concentration and accumulation of newly synthesized CAs under the experimental conditions of our behavioral tests A second objective was to resolve several major discrepancies which had been reported on the effects of protein synthesis inhibitors (PSIs) on the synthesis of catecholamines [11, 19, 33, 39] The conclusions drawn from the behavioral experiments will depend on what effect PSIs have on catecholamine levels and/or turnover

The rationale for the behavioral experiments was that if ANI acts on memory formation chiefly by its secondary action on CAs, then one should be able to substitute catecholamine synthesis inhibitors (CAIs) for ANI and obtain similar amnesia Three CAIs having major but different effects on tyrosine, NE and DA were tested along with ANI in a series of experiments that allowed comparison of the behavioral effects Failure to observe a reasonably close correspondence of behavioral effects would support the hypothesis that protein synthesis is a necessary component of long-term memory trace formation

BIOCHEMICAL EXPERIMENTS

METHOD

Subjects

CD-1 male mice obtained from Charles River Breeding Laboratories, Wilmington, MA, at 6 weeks of age were used in both biochemical and behavioral experiments at 60–80 days of age Mice were housed singly 24 hr prior to training and until the end of the experiment In the biochemical experiments, mice were fasted during the 24 hr isolation to reduce the variability of tyrosine in brain tissue

Chemicals and Drug Administration

L-[14C(U)]-tyrosine (500 mCi/mmole) and L-[14C(U)]-valine (300 mCi/mmole) were obtained from New England Nuclear, Boston MA The scintillation counting cocktail was obtained from Research Products International Mt Prospect IL

The dose and source of protein and catecholamine synthesis inhibitors were anisomycin (ANI, 20 mg/kg), Pfizer Diagnostics, Clifton, NJ (now obtainable from Warner-Lambert, General Diagnostics Division, Morris Plains, NJ), cycloheximide (CYCLO, 150 mg/kg) and diethyldithiocarbamic acid (DDC, 250 mg/kg), Sigma Chemical Co , St Louis, MO, alpha-methyl-p-tyrosine (AMPT 160 mg/kg), ICN-Nutritional Biochemical Co , Cleveland OH tetrabenazine (TB, 10 mg/kg), Hoffman-La Roche, Nutley, NJ, d-amphetamine (2 0 mg/kg) was obtained from commercial sources through UCLA Pharmacy The doses of DDC and AMPT were the highest possible that did not impair the subject s motor ability TB was not used in paradigms calling for pretraining injections as it rendered the mice untrainable All drug and control saline injections were administered subcutaneously at pH 6 0 to 6 5 except for TB which was adjusted to pH 5 0 to 5 5 All solutions were blind-coded to eliminate experimenter bias

General Description of Procedure

Numerous procedures are published for the determination of catecholamines Many of the methods are variants of basic procedures published by Anton and Sayre [3,4] After careful evaluation of alternatives, the following procedures were adopted as best suiting our needs

To determine turnover of DA and NE, L-[14C(U)]-tyrosine (5 μCi) in saline (100 μl) was injected intraperitoneally 10 min prior to sacrifice The times from drug administration to sacrifice are shown in the tables Animals were sacrificed by cervical dislocation, brains (including cerebellum and medulla) were rapidly excised, weighed, frozen on dry ice, and stored on dry ice overnight

Tyrosine was separated from NE and DA on microcolumns at room temperature following the procedures described by Barchas and co-workers [5] A mouse brain was homogenized in a Potter Teflon-glass homogenizer for 1 min in 3 0 ml of ice-cold 0 4 N $HClO_4$ plus 0 1 ml 10% EDTA The homogenate was centrifuged at 30,000 $\times$ g for 15 min and the precipitate washed with 2 0 ml of 0 4 N $HClO_4$ and recentrifuged The wash was combined with the first supernatant and the combined supernatants were adjusted to pH 6 00±0 15 by the careful addition of 3 N KOH Since the catecholamines are destroyed rapidly under alkaline conditions, the neutralization was carefully controlled using an autotitrator At 4°C, the precipitated $KClO_4$ was removed by centrifugation, and the volume of supernatant adjusted so that 1 ml was equivalent to 75±5 mg of initial brain weight

Microcolumns containing Fisher Rexyn 102 (instead of Bio-Rex 70) were prepared following the description of Holman *et al* [36] Immediately prior to use, a column was washed with 1 5 m of 0 1% EDTA The neutralized extract from the brain was placed on the column Tyrosine was not retained, but to insure complete removal the column was washed with 1 5 ml of 0 02 M sodium phosphate buffer, pH 6 5 containing 0 2% EDTA, followed by a 1 5 ml wash of 0 1% EDTA At least 99% of added tyrosine was recovered in the column loading effluent plus washes Total tyrosine and radioactive tyrosine was determined in this fraction 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 DA were recovered Gentle pressure was applied with N_2 gas to remove all liquid from the column and to provide an oxygen-free atmosphere for the catecholamines If the concentrations of CAs were to be determined, the extracts were stored in the dark at −20°C under N_2 until analyzed

We used assays developed by Anton and Sayre [3] to determine DA and NE Fluorescence of the dihyroxyindole derivative of DA was measured with excitation at 316 nm and emission at 370 nm in a Perkin-Elmer Fluorescence Spectrophotometer Model MPF2A 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

NE was converted to 3,5,6-trihydroxyindole with potassium ferricyanide and alkaline ascorbate [3] The emission fluorescence due to excitation of indole at 409 nm was measured at 519 nm 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 was determined as described by Udenfriend [51] by the formation of a phenoxazinone derivative from tyrosine and nitrosonaphthol in the presence of nitrate-nitrite catalyst Tyrosine standard (5 μg/ml) was prepared in H_2O by dissolving in a small volume of dilute KOH and then adjusting the pH to 9 5 using HCl Useful standards ranged from 0 5 to 3 5 μg tyrosine, a linear response was obtained Fluorescence of phenoxazinone in the aqueous phase was determined by emission at 570 nm upon excitation at 460 nm The fluorescence of appropriate internal standards as well as blanks was also determined

To determine radioactive tyrosine, 1 ml of the combined loading effluent plus washes was counted in a glass minivial with the addition of 4 5 ml RPI counting cocktail 3a70B in a Beckman model LS9000 liquid scintillation counter To determine radioactivity in the CA fraction, the HOAc effluent from the Rexyn column was collected in a glass maxivial and counted as above, adding 12 ml RPI counting cocktail

In evaluating the experimental determinations of the conversion rate of tyrosine to DA and NE, the limitations of the method employed must be considered The conversion rate is based upon the radioactivity in the catecholamines and the average specific activity of the labeled tyrosine during the labeling interval In order to significantly label the small pool of cerebral CA, a relatively long time interval is required However, the time interval for the labeling of the CA is constrained by two factors (1) the specific activity for tyrosine in brain increases linearly for only 15–20 min after administration of tyrosine and then falls rapidly (Fig 2), and (2) for labeling periods longer than 10–15 min, significant radioactivity will be lost from the CA due to their further metabolism With the exception of the study by Bloom *et al* [11], where a 30-min incorporation period was used, the labeling period in most studies was limited to 15 min or less This relatively brief labeling period has its own drawback, however, since during this period less than 0 4–0 5% of the administered tyrosine will be found in brain, and only about 0 25% of the label from the brain tyrosine will be present in the CA fraction in the normal animal Thus, in our experiments, only 100–200 dpm was normally present in the brain CA fraction 10 min after administration of 5 μCi of 14C-

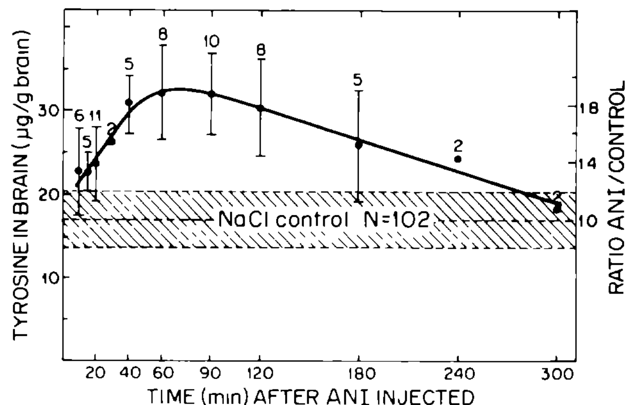

FIG 1 The effect of ANI (20 mg/kg) on brain tyrosine concentration The shaded area indicates the tyrosine concentration and standard deviation of saline injection control mice (NaCl) The number of animals and standard deviation for each data point are indicated

tyrosine In order to avoid erroneously high answers, this fraction must be completely separated from any residual contaminants, especially tyrosine Although other investigators have used large amounts of 3,5-3H-tyrosine in their studies, the effective dpm anticipated in the CA fraction is reduced by three factors (1) loss of one 3H in the conversion of 3,5,-3H-tyrosine to CA, (2) lower efficiency obtained in counting 3H compared with 14C, typically by at least a factor of two, and (3) higher background for tritium samples compared to 14C

The other important parameter in the estimation of catecholamine synthesis is the average specific activity of the precursor tyrosine during the labeling period This value is determined by the average concentration of the cerebral tyrosine and the rates of uptake and utilization of the labeled tyrosine In the control animals, the concentration of tyrosine is assumed not to change, consequently the average concentration is taken to be the same as the final concentration However, when PSIs are used, *the concentration of cerebral tyrosine is changing*, especially shortly after administration, and the average value may be as much as 10% less than the final value We made a correction to account for this change varying from 10% over the first 10 min after administration of the PSI to 4% for animals sacrificed at 40 min No correction was made at later time points where the rate of change was less rapid Bloom, Quinton and Carr [11] and Lundgren and Carr [39] did not specifically address this issue, presumably the tyrosine concentration at the time of sacrifice was used in the calculation of specific activity

The inhibition of protein synthesis by ANI, CAIs, and a combination of ANI plus a CAI was determined in most experiments using L-[14C(U)]-valine (5 μCi) as the protein precursor [14,24] Protein synthesis inhibition was determined in selected experiments following the same general procedures using L-[14C(U)]-tyrosine (5 μCi) as the precursor For the latter experiments, percentage inhibition of protein synthesis was calculated using the final specific activity of tyrosine for each experimental animal The incorporation time was 10 min

TABLE 1

EFFECTS OF ANI ON CEREBRAL CATECHOLAMINE CONCENTRATION

Injection Schedule	Dose mg/kg	N	Sacrifice Time* (hr)	Percent of Control† DA	NE
ANI	20	7	0 25–1 50	110 ± 17	97 ± 11
ANI	40	3	1	105 ± 6	98 ± 5
ANI	70	3	1 50	112 ± 10	98 ± 7
ANI+ANI+ANI	20‡	9	5	92 ± 10	83 ± 6

*Sacrifice time is the time after the initial injection of ANI

†The control value for dopamine was 1 16 ± 0 2 μg/g and for norepinephrine was 0 29 ± 0 02 μg/g These values are comparable to those reported by Goodman, Flexner and Flexner [33] but are approximately one-half of the values reported by Bloom, Quinton and Carr [11] The results given are the means and standard deviations

‡Three injections of ANI were administered at two-hour intervals The dose is for each injection

TABLE 2

EFFECT OF CAIs ON TYROSINE AND CATECHOLAMINE CONCENTRATIONS

Drug	Dose mg/kg	N	Sacrifice Time (min)	Percent of Control* Tyrosine	DA	NE
DDC	250	1	15	140	—	—
	250	1	30	160	—	—
	250	8	60–120	115 ± 21	97 ± 6	60 ± 8
	1000	3	60	180 ± 14	80 ± 3	48 ± 2
TB	10	1	15	101	—	—
	10	1	30	117	—	—
	10	2	60	93 ± 6	11 ± 8	30 ± 10
	10	1	120	98	—	—
AMPT	160	8	60	—	77 ± 21	84 ± 11
	160	2	120	—	40 ± 7	68 ± 8

*Results are the mean and the standard deviation

RESULTS

Effects of Anisomycin or Cycloheximide on Tyrosine and Catecholamine Concentrations

An extensive series of experiments was performed to determine the effects of ANI on brain concentration of tyrosine A single injection of ANI (20 mg/kg) caused a gradual increase in cerebral tyrosine concentration which peaked about 75 min later at approximately double the normal value and was followed by a slow decline over the next few hr, returning to the baseline about 5 hr after injection (Fig 1) Repeated doses of ANI extended the duration of the effect but did not increase the maximum concentration of tyrosine Higher doses of ANI (120 mg/kg) extended the duration slightly and increased the maximum concentration at 2 hr to about 225% of control CYCLO (150 mg/kg) caused increases in cerebral tyrosine concentration similar to a single injection of ANI The effect of ANI on DA or NE content was investigated Neither DA nor NE content was found to change significantly as a result of ANI administration (Table 1)

Effects of Catecholamine Inhibition on Tyrosine and Catecholamine Concentrations

A second series of experiments investigated the effects of DDC and TB on tyrosine concentration, and of DDC, TB, and AMPT on catecholamine concentrations (Table 2) DDC (250 mg/kg) caused an increase in cerebral tyrosine concentration, but this increase was not as pronounced as that caused by ANI and was of shorter duration TB (10 mg/kg) caused little or no change in cerebral tyrosine concentration The effect of AMPT on tyrosine concentration was not determined due to its interference in the tyrosine assay DDC caused a 40% decrease in NE concentration, while TB drastically lowered the concentration of both DA and NE AMPT caused approximately 50% reduction in catecholamines 2 hr after administration

TABLE 3

EFFECTS OF COMBINATIONS OF ANI AND CAIs ON TYROSINE AND CATECHOLAMINES*

Drug	Dose mg/kg	N	Sacrifice Time (min)	Percent of Control Tyrosine	DA	NE
DDC	250	1	15	180	—	—
	250	1	30	300	—	—
	250	2	60	190 ± 14	90 ± 13	49 ± 10
	250	5	60	195 ± 28†	—	—
	250	1	120	270	—	—
TB	10	1	15	118	—	—
	10	1	30	145	—	—
	10	2	60	146 ± 10	10 ± 8	40 ± 9
	10	5	60	140 ± 7†	—	—
	10	1	120	125	—	—
AMPT	160	2	60	—	66 ± 8	65 ± 7

*ANI was administered at 20 mg/kg, DDC, TB or AMPT was administered one hr later The sacrifice time is the time after administration of the catecholamine inhibitor

†For separate groups of mice, tyrosine was determined by the direct method The N was 5 for each group The result is given as the mean and standard deviation

Effects of Combined Administration of Anisomycin and a Catecholamine Inhibitor on Tyrosine and Catecholamine Concentrations

A third series of experiments investigated the effects of administration of combinations of ANI followed by a CAI on tyrosine and catecholamine concentrations The combined action of ANI and DDC caused higher levels of brain tyrosine

TABLE 4A

INHIBITION OF PROTEIN SYNTHESIS BY ADMINISTRATION OF CAI AND ANI USING L-[U-14C]-VALINE AS PRECURSOR*

Drug Group†	Injection Time (hr)	Sacrifice Time (hr)	Inhibition of Protein Synthesis (%)
ANI+ANI+ANI	0, 2, 4	4 25	94 ± 3
DDC+DDC+DDC	0, 2, 4	5	43 ± 5
AMPT+AMPT+AMPT	0, 2, 4	5	18 ± 8
TB+TB+TB	0, 2, 4	5	38 ± 4
ANI+Saline	0, 1	2	63 ± 4
ANI+DDC	0, 1	2	78 ± 3
ANI+AMPT	0, 1	2	66 ± 3
ANI+TB	0, 1	2	68 ± 2

*Inhibition was based upon the ratio of TCA insoluble activity to the total activity of supernatant and precipitate of experimental and saline-injected control mice The time of sacrifice is measured from the first drug injection Three mice were used for each drug group and six mice for the control group

†Injections of the CAIs and ANI were at 1-hr or 2-hr intervals The dose of each injection was ANI, 20 mg/kg, DDC, 250 mg/kg, TB, 10 mg/kg, and AMPT, 160 mg/kg In the experiments employing ANI and a CAI (Table 4A and 4B), ANI was given at 0 hr and the CAI at 1 hr later

TABLE 4B

INHIBITION OF PROTEIN SYNTHESIS BY ADMINISTRATION OF A CAI AND ANI USING L-[U-14C]-TYROSINE AS PRECURSOR‡

Sacrifice Time (hr)	N	ANI % Inhibition	N	ANI+DDC % Inhibition	N	ANI+TB % Inhibition
<1	12	76 ± 11	3	81 ± 4	2	78 ± 3
1 or 1 5	12	79 ± 8	2	78 ± 3	2	80 ± 4
2	6	72 ± 8	1	72	1	81
3	7	46 ± 10				
4	2	33 ± 9				

‡Inhibition was based on TCA-insoluble radioactivity and specific activity of TCA-soluble tyrosine of individual mice Results are given as the mean and standard deviation

than either drug alone (Table 3) ANI + TB resulted in tyrosine levels that were elevated, but less than those obtained after administration of ANI alone For catecholamines, not unexpectedly, in each case the action of the CAI prevailed and the resulting concentrations of the CA were comparable to those obtained with the CAI alone For example, when DDC alone was administered, the DA concentration was not significantly reduced, whereas NE concentration was reduced by approximately 50% The combination of ANI and DDC did not cause a further reduction of either DA or NE TB caused major reductions of both DA and NE, and comparable reductions were found when ANI plus TB were administered in combination

Effects of Anisomycin and Catecholamine Inhibitors on Protein Synthesis

In the first series of experiments to study protein synthesis inhibition, the effects on protein synthesis of a series of three injections of CAIs at 2 hr intervals were compared to the effects on an equivalent series of ANI injections, using L-[14C(U)]-valine as the precursor In addition, the combined effect of ANI plus a CAI on protein synthesis was determined Using 14C-valine as a precursor, DDC was the most effective inhibitor of protein synthesis, but the apparent inhibition was less than one-half of that obtained from ANI (Table 4A) Any combination of ANI and a CAI caused only a slight increase in inhibition of protein synthesis over that caused by ANI alone

In conjunction with the experiments to determine the effects of ANI and CAIs on catecholamine synthesis, we studied inhibition of protein synthesis using L-[U-14C]-tyrosine as a precursor (Table 4B) In these studies, a correction can be made for the specific activity of the tyrosine precursor As a result, the calculated inhibition was slightly less than when inhibition was based on total TCA soluble activity, due to the increase in free tyrosine and the resultant decrease in precursor specific activity No addi-

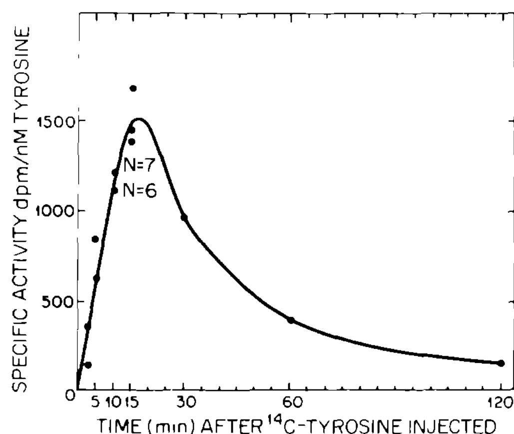

FIG 2 Specific activity of 14C-tyrosine in mouse brain as a function of time after IP injection Each point represents the value for a single mouse brain except for two 10-min points indicated as the average of 7 brains or 6 brains, respectively, in two separate experiments

tional effect was found on protein synthesis in combinations of DDC or TB with ANI A single injection of DDC or TB caused little if any protein synthesis inhibition (data not shown)

Effect of Anisomycin and of Catecholamine Inhibitors on Uptake of 14C-Tyrosine Into Mouse Brain

Since the determination of CA synthesis rate depends upon both the brain concentration of tyrosine and the tyrosine specific activity, the uptake of 14C-tyrosine was determined for individual mice The upatke in brain 10 min after tyrosine administration was approximately 0 4–0 5% of the administered tyrosine Other experiments in which mice were sacrificed at intervals ranging from 2 5 min to 60 min after administration showed the total amount of free radioactive tyrosine in the brain increased almost linearly for the first 15–20 min after administration, and then declined rapidly (Fig 2)

As summarized in Table 5, neither the protein synthesis inhibitors nor CAIs alone or in combination had a large effect on uptake of 14C-tyrosine into brain In fact, when allowance was made for the amount of labeled tyrosine incorporated into protein (about 14% of the total uptake when protein synthesis is not inhibited), little or no drug effect on uptake was found It should be noted that the uptake of tyrosine was quite variable among mice However, since values for protein synthesis or catecholamine synthesis rate are calculated based on tyrosine specific activity of each individual mouse, this variability should not markedly influence the determination of these measures

Effect of Anisomycin and of Catecholamine Inhibitors on Conversion of Tyrosine to Dopamine and Norepinephrine

The effect of ANI, CYCLO and the CAIs on the conversion of 14C-tyrosine to the combined pool of DA and NE was evaluated at different time periods after drug administration The results tabulated in Table 6 show that the PSIs have only a moderate effect on the conversion rate of 14C-tyrosine into DA and NE We were unable to discern a time

TABLE 5

UPTAKE OF 14C-TYROSINE BY MOUSE BRAIN

Drug Treatment	N	14C-Tyrosine Uptake nCi/g brain	Ratio Drug/Saline
Saline	50	44 ± 11	—
ANI (20 mg/kg)	43	49 ± 20	1 11
ANI (120 mg/kg)	10	57 ± 16	1 30
CYCLO (150 mg/kg)	12	46 ± 16	1 05
DDC (250 mg/kg)+Saline	6	45 ± 7	1 02
DDC (250 mg/kg)+ ANI (20 mg/kg)	6	43 ± 9	0 98
TB (10 mg/kg)+Saline	6	44 ± 12	1 00
TB (10 mg/kg)+ ANI (20 mg/kg)	5	46 ± 22	1 05

*The 14C-tyrosine uptake into brain was determined 10 min after the intraperitoneal administration of 5 μCi of L-[14C]-tyrosine and is based upon the amount of radioactivity present in the soluble fraction When protein synthesis is not inhibited, about 15% additional [14C]-tyrosine is present in the TCA insoluble fraction Results are given as the mean and standard deviation

dependence of protein synthesis inhibitors on conversion of tyrosine to CA over a 4-hr period after drug administration The inhibition of conversion by ANI (20 mg/kg) or CYCLO (150 mg/kg) was about 20% Even ANI at 120 mg/kg reduced the conversion by only 30%

The effect of DDC or TB with and without ANI on tyrosine conversion was determined No effect of DDC (250 mg/kg) on the conversion to DA and NE combined was found This was as anticipated since DDC primarily modified NE and not DA and about 80% of the apparent conversion of tyrosine into catecholamines is into DA in a precursor pulse experiment of short duration TB (10 mg/kg) produced approximately 80% inhibition of tyrosine conversion into the combined catecholamines 10 to 20 minutes after administration This inhibition was of short duration as the conversion rate was approximately 80% of controls at 60 minutes ANI in combination with either DDC or TB had no additional effect on the conversion rate

DISCUSSION

The most frequent alternative explanation advanced for the biochemical basis of the amnestic action of protein synthesis inhibitors is that they act by impairing catecholaminergic action [20, 31, 33] Biochemical evidence in support of this view comes primarily from experiments of Flexner and co-workers [19, 20, 33] and Carr and co-workers [11,39] In brief, the following actions with respect to the catecholamine system have been attributed to protein synthesis inhibitors (1) a large increase in cerebral tyrosine concentration, (2) inhibition of tyrosine hydroxylase, (3) marginal changes in the concentrations of the catecholamines DA and NE, both increases and decreases being reported, and most important, (4) drastic reductions in the rate of synthesis of the catecholamines, DA and NE

In the discussion which follows, the reported indirect effects of PSIs, primarily ANI and CYCLO, will be evaluated

and compared with those of the CAIs (DDC, TB, and AMPT) Our conclusions with respect to the biochemical actions of these drugs are as follows (1) PSIs produced an increase of 100% or more in cerebral tyrosine concentration, DDC produced a moderate increase, and TB a small increase (2) The inhibition of tyrosine hydroxylase by PSIs is probably 10% or less (3) PSIs produced little or no change in cerebral CA concentrations, whereas CAIs produced large changes in one or more of the catecholamines (4) Due to methodological problems, it is difficult to determine inhibition of catecholamine synthesis by PSIs, however, we found that no more than 20% inhibition was caused by PSIs This relatively small inhibition is consistent with the observation that CA concentrations are not reduced by PSI administration The reduction of CA synthesis by PSIs was markedly less than the inhibition by known CAIs (5) When a PSI and a CAI are both administered, the predominant pharmacological effect of each is observed, independent of the other

Cerebral Tyrosine Concentration

All investigators agree that protein synthesis inhibition leads to at least an 80–100% increase in the brain levels of tyrosine This increase is probably primarily due to its continued uptake and/or formation in the brain without the concomitant utilization for protein synthesis Maximum levels are reached after approximately one hour, and a slow return to normal levels occurs as protein synthesis recovers We found that the variability of cerebral tyrosine concentration among individual mice was relatively large, especially in mice given ANI Selective increases in cerebral concentrations of a number of other amino acids would also result from the protein synthesis inhibition ([45], unpublished data,* Flood and Bennett)

As has been previously reported by Bloom, Quinton, and Carr [11], we found that DDC produced a moderate but short increase in cerebral tyrosine TB was less effective in this respect We did not determine the action of AMPT, however, Flexner and Goodman [19] reported only a minimal increase in cerebral tyrosine after AMPT administration

Inhibition of Tyrosine Hydroxylase by Protein Synthesis Inhibitors

Flexner *et al* [20] reported that mice previously treated with CYCLO had about 20% less tyrosine hydroxylase activity than saline injected animals However, CYCLO added to the assay medium did not inhibit tyrosine hydroxylase These authors concluded that the V_{max} of the CYCLO-exposed brain was reduced but not the Km towards either CYCLO or the pterine cofactor Using a larger dose of CYCLO, Markey and Sze [40] found 35–45% inhibition of the hydroxylase They reported that the Km of the pterine cofactor was increased by cycloheximide treatment In agreement with Flexner *et al* [20], Markey and Sze found that CYCLO added in vitro did not influence tyrosine hydroxylase activity

Squire *et al* [50] investigated the inhibition of tyrosine hydroxylase by two different assay methods Using the assay method used by Flexner *et al* [20], they found only 11% inhibition of the hydroxylase after CYCLO treatment and 8% after ANI They concluded that a portion of this low inhibition was an artifact due to a greater dilution of the labeled tyrosine used in the assay by endogenous tyrosine present in inhibitor-treated mice While greater inhibition was found by an alternative enzyme assay, the authors concluded that the higher apparent inhibition was most probably an artifact due to large precursor dilution in the assays of the inhibited animal Squire *et al* showed that AMPT inhibited tyrosine hydroxylase to a greater degree than did either CYCLO or ANI, but was not an effective amnestic agent We did not directly determine the inhibition of tyrosine hydroxylase by ANI or CYCLO but would conclude that it is no more than 20%, and probably less than 10%

Concentrations of Catecholamines

Goodman *et al* [33] reported that acetoxycycloheximide (AXM) caused an increase of 20–40% in cerebral catecholamines for several hours after its administration followed by a decrease of nearly 40% at 17 hr after administration Flexner *et al* [20] using a smaller dose of AXM reported a 20% decrease in NE concentration from 4 to 17 hr later but no significant increase in concentration was observed at any time CYCLO caused moderate (10–30%) increases in NE and DA one hour after administration, but no significant changes were reported at later times No change was reported as a result of ANI administration Bloom *et al* [11] investigated the changes in NE and DA over a 6 5 hr period after administration of CYCLO, and reported only one significant increase of 23% in the concentration of DA, at 3 5 hr This group did not report the effects of ANI on catecholamine concentration Freedman *et al* [31] have also concluded that CYCLO does not modify the concentration of DA or NE between 0 75 hr and 24 hr after administration, whereas DDC reduces the concentration of NE by 30–40% between 2 and 4 hr after administration and does not modify DA concentration (These investigators also reported that both ANI and CYCLO inhibited K^+ stimulated release of NE in vitro but only at high concentrations of CYCLO or ANI) We did not find any significant change in the concentration of CAs following ANI administration, this was also the case even after a series of ANI injections given at 2 hr intervals On the other hand, as expected, we found the DDC lowered cerebral NE by about 50% but did not significantly modify DA concentration TB decreased drastically (70–90%) both DA and NE in brain, while AMPT produced moderate reduction of both DA and NE When ANI was combined with a CAI the action of the CAI prevailed We conclude, therefore, that protein synthesis inhibitors do not produce large changes in catecholamine concentrations, especially when compared with the changes produced by well known CAIs

Rate of Synthesis of Dopamine and Norepinephrine

Goodman *et al* [33] reported that intracerebral administration of AXM in mice produced a severe and long-lasting depression of cerebral catecholamine synthesis The minimum synthetic rate of about 10% of normal was reached 3 hr after administration, and in the case of NE the synthetic rate remained depressed for 24 hr In a subsequent report, Flexner and Goodman [19] reported that the accumulation rate of DA was reduced by 65%, but they found only 35% (non-significant) reduction in the rate of NE synthesis CYCLO led to a reduction of up to 50–60% in the accumulation rate of NE and DA, and this inhibition persisted for at least 12 hr ANI, 2 hr after administration, produced a comparable inhibition (50–60%) of both NE and DA synthesis, data for longer time intervals were not reported

Bloom *et al* [11] reported inhibition of NE and DA syn-

TABLE 6

EFFECT OF ANI OR CYCLO ON TYROSINE CONVERSION TO DOPAMINE AND NOREPINEPHRINE*

Time (min)	Saline %	ANI 20 mg/kg %	ANI+ANI 20 mg/kg %	ANI 120 mg/kg %	CYCLO 150 mg/kg %
10–20	0.56 ± 0.10 (14)	79 ± 11 (5)	79 ± 7 (2)	68 ± 25 (3)	86 ± 16 (3)
40–60	0.63 ± 0.06 (8)	84 ± 19 (4)	73 ± 14 (3)	56 (1)	—
90–120	0.61 ± 0.13 (10)	70 ± 14 (8)	82 ± 18 (2)	67 ± 10 (2)	80 ± 6 (3)
180–240	0.54 ± 0.11 (3)	100 ± 24 (6)	146 ± 15 (2)	80 ± 13 (3)	87 ± 24 (5)

*The rate was measured 10 min after IP injection of L-[14C(U)]-tyrosine. The values for the saline-injected mice are μg DA+NE synthesized/hr/g brain (mean ± standard deviation). Values for the drug-injected mice are expressed as the mean percentages of the control ± the standard deviation. The numbers in parentheses are the number of mouse brains assayed. Time is measured after the last drug administration.

thesis of 75–90% over the first 3–5 hr after CYCLO administration, while Lundgren and Carr [39] reported about 65% inhibition of both NE and DA synthesis 2 hr after ANI administration. Bloom *et al.* [11] also found that DDC caused nearly complete inhibition of NE synthesis for at least 2 hr after administration with recovery to about 50–60% of normal rate during the next 4–5 hr. DA synthesis was reduced to a minimum of 60% of controls 1½ hr after administration of DDC, but recovered to nearly normal 5 hr later. It should be noted that the synthetic rates of CA of control mice reported in these two papers differ by a factor of 6 to 10.

In view of the limited data concerning the effects of ANI on CA synthesis reported by these investigators, a principal objective of the present biochemical study was to investigate the effects of ANI on this synthetic rate. A large number of time intervals was studied from 10 min after drug administration up to 5 hr after the initial dose (ANI+ANI, 180 min, Table 6).

During this time interval ANI at the 20 mg/kg dose inhibited CA synthesis by an average of 15–20%. As is apparent from the data presented in Table 6, the variability in the determined CA synthetic rate was relatively large (coefficient of variation was approximately 20%). It should be noted that coefficients of variation of 35% or more were frequently observed for these same measures by previous investigators. Consequently, we do not feel it is possible to discern a valid time course for the inhibition of CA synthesis from the available data. CYCLO (150 mg/kg) produced a moderate inhibition of CA synthesis, comparable to that of ANI. The inhibition produced by a large dose of ANI (120 mg/kg) was about 30%. In view of the experimental limitations of the method, which are discussed more fully under the Method section, the conversion rates reported by Flexner and Goodman [19] and Goodman, Flexner and Flexner [33] and those we found are in fair agreement.

In limited studies using DDC, we did not find a significant reduction in the conversion of tyrosine to the combined catecholamines. Since the main effect of DDC is on NE, and NE comprises only about 20% of the combined catecholamines, this result is expected. On the other hand, TB produced a marked (80%) but relatively short-acting inhibition of the conversion of tyrosine into both DA and NE, as anticipated. ANI did not modify this inhibition.

Uptake and Utilization of Tyrosine

As noted earlier, the uptake of labeled tyrosine is approximately linear for about 15 min after its administration. Linear uptake of labeled tyrosine was also found in mice administered ANI. However, little or no tyrosine was used for the diminished protein synthesis in mice administered ANI. Consequently, the amount of free radioactive tyrosine in inhibited animals was 5–30% greater than in control mice (Table 5). Nevertheless, due to the large increase in free cerebral tyrosine when protein synthesis is inhibited, the average specific activity of the cerebral tyrosine is frequently 50% or less than that of the control. Goodman *et al.* [33] and Flexner *et al.* [20] did not address the effects of inhibitors on tyrosine uptake. However, Bloom *et al.* [11] noted that CYCLO (as well as DDC, and DDC plus amphetamine) all significantly elevated the specific activity of the precursor tyrosine by a factor up to 6 to 7 times that of the control mice, up to 3.5 hr after drug administration. This elevation of tyrosine specific activity occurred together with an increase in the amount of tyrosine present. It can be calculated that Bloom *et al.* [11] report 10 times as much soluble labeled tyrosine in the CYCLO-treated mice during the interval from 0.5 hr to 3.5 hr after drug administration as in the controls. Although no data are given, Lundgren and Carr [39] reported that the specific activity of 3H-tyrosine was increased by all drug [ANI] treatments at 2 hr due to a greater increase in 3H-tyrosine concentration than in endogenous levels of tyrosine. Since the tyrosine level of the experimental animals at this time was a minimum of 175% of the controls, this suggests that they found at least 100% increase in labeled tyrosine uptake. This unexplained discrepancy between their results and ours regarding the effects of PSIs on the precursor tyrosine specific activity may be the basis for our divergent conclusion regarding the effects of PSIs on catecholamine synthesis. Failing to consider the effect of changing precursor pool size results in a significant overestimation of CA inhibition.

Inhibition of Protein Synthesis

The present study confirmed that ANI is an effective inhibitor of brain protein synthesis. Of the CAIs tested, DDC is the most effective inhibitor of protein synthesis, but even

three consecutive doses of DDC produced less than 50% inhibition Bloom *et al* [11] also reported that DDC produced moderate inhibition of protein synthesis The combination of ANI and a CAI produced about the same inhibition as ANI alone

Summary of Biochemical Studies

These studies have confirmed that the predominant biochemical effect of ANI is the inhibition of protein synthesis, with relatively minor effects on catecholamine metabolism On the other hand, the predominant effects of the CAI are, as expected, on concentrations and synthesis rates of catecholamines, with relatively minor effects on protein synthesis When combined, the predominant effect of each drug is observed, that is, the inhibition of protein by ANI is not significantly modified by CAIs, and the inhibition of CA synthesis by the CAIs is not markedly changed by the presence of ANI

BEHAVIORAL EXPERIMENTS

Rationale

The hypothesis that inhibitors of protein synthesis affect memory retention by inhibiting the synthesis of CA neurotransmitters [19] should predict the CAIs and PSIs would have similar effects on memory retention test performance and that one should be able to substitute CAIs for PSIs and obtain similar results Assuming a unitary theory of memory processing, the hypothesis that PSIs and CAIs cause amnesia by different mechanisms [16,47] predicts that one cannot substitute CAIs for PSIs and obtain similar results

METHOD

The mice and chemicals are as described in the Biochemical Experiments

Step-Through Passive Avoidance

The procedure for training and testing mice for the one-trial, step-through passive avoidance task was described previously [24] In brief, the apparatus consisted of a black start compartment joined to a white shock compartment by a partition containing a mousehole through which the mice could enter the white compartment In the white compartment, footshock was given until the mouse returned to the black compartment Acquisition is influenced by the latencies-to-enter and latencies-to-escape the shock compartment and by the footshock intensity Only mice entering in two or three sec and escaping in two or three sec were used, the use of these narrow performance criteria has proved successful in reducing between-subject variability On the average, we reject less than 15% of the mice To test retention, each mouse was again placed into the black compartment and the time required for the mice 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 since this represents the range of latencies-to-enter of experimentally naive mice singly housed for one week Percent forgetting is defined as the percentage of the mice having an entry time at testing of 20 sec or less Most trained non-amnestic mice did not enter the white compartment within the three-minute test period Training and testing were done between the hours of 0700 and 1400

T-Maze Active Avoidance

The T-maze apparatus and training were described previously [23] The apparatus consisted of a black plastic start alley with a start box at one end and two goal boxes at the other, a brass grid floor ran throughout the entire maze Each goal box was fitted with a slotted, transparent plastic liner (the bottom of which went below the shock grid) which was used to remove the mice from the goal box The start box was separated from the start alley by a black plastic guillotine door which prevented the mouse from moving down the start alley until training started The conditioned stimulus was a doorbell-type buzzer The mice were given 5 training trials, the intertrial interval was about 45 sec

Mice were not permitted to explore the maze prior to training A training trial started with a mouse placed into the start box The guillotine door was then raised and the buzzer sounded simultaneously Five sec later footshock was applied The goal box that the mouse first entered on this trial was designated as "incorrect" and the footshock was continued until the mouse entered the other goal box, which on all subsequent trials was designated "correct" for the particular mouse At the end of each trial, the mouse was removed from the goal box by lifting the plastic liner and carefully returned to its home cage A new trial began by raising the guillotine door, with footshock beginning 5 sec later if the mouse had not moved into its correct goal box As training proceeded, a mouse could make one of two types of responses If a mouse failed to reach the correct goal box in 5 sec or less it was considered an escape, otherwise, it was classed an avoidance response

Statistical Evaluation

Because step-through passive avoidance test latencies in drug and saline injected mice are bimodally distributed [24], a measure of central tendency such as the mean or mode would be an inappropriate descriptive statistic In passive avoidance experiments, significance levels were determined by the Chi-Squre Test using the number of mice classed as forgetting or remembering original training When the N for any one of the four theoretical frequencies was less than 7, the Fisher Exact Probability Test [35] was used to obtain the *p* value In Experiment 5, T-maze active avoidance was used and distribution of the number of trials to make the first avoidance response was approximately normal To facilitate comparison with the other experiments, the percent forgetting score was included

EXPERIMENT 1 EFFECT OF TRAINING FOOTSHOCK INTENSITY ON THE EFFECTIVENESS OF AMNESTIC AGENTS

The purpose of this experiment was to compare the amnestic effectiveness of a 60-min pretraining injection of AMPT (160 mg/kg) or DDC (250 mg/kg) with that of a 15-min pretraining injection of ANI (20 mg/kg) at two footshock intensities (0 32 and 0 36 mA) The one-trial passive avoidance responding (PAR) task was used The N per group was 20 The retention test was given one week after training

It was found that at the lower footshock intensity both CAIs and ANI caused a significantly greater percentage of amnesic mice than did a saline injection ($p<0$ 01) At the higher footshock intensity, neither CAIs nor ANI yielded retention scores that differed significantly from the saline-injected group (Table 7) This confirmed our previous result that as the training strength increases, the effectiveness of an amnestic treatment decreases [24] However, this experi-

TABLE 7

AMNESTIC EFFECT OF CA AND PROTEIN SYNTHESIS INHIBITORS AT TWO TRAINING FOOTSHOCK INTENSITIES

Drug Treatment	Forgetting (%) Footshock Intensity 0 32 mA	0 36 mA
Saline	20	20
DDC	75	20
AMPT	85	20
TB	70	25
ANI	75	20

The values are the percent of mice classed as forgetting original training Subjects were trained on the one-trial passive avoidance task at 2 different shock intensities The CAIs and saline were administered 60 min prior to training, ANI was administered 15 min prior to training Retention was tested one week after training N=20 per group

TABLE 8

PERCENT AMNESIA AS A FUNCTION OF THE INTERVAL BETWEEN DRUG ADMINISTRATION AND TRAINING

Drug	N	Interval 0 25 hr	N	Interval 1 hr	N	Interval 2 hr
Saline	20	25	20	20	17	24
ANI	15	73	14	64	18	33
DDC	14	57	12	58	14	64
AMPT	15	27	13	54	15	67

The values are the percent of mice classed as forgetting original training Shock intensity was 0 34 mA Drug was administered to separate groups of mice 0 25, 1, or 2 hr prior to training Mice were tested one week after training

ment did not provide evidence that the effective mode of action of CAIs and PSIs differed

EXPERIMENT 2 COMPARISON OF EFFECTS OF ADMINISTRATION OF MULTIPLE DOSES OF CAI OR ANI ON MEMORY RETENTION TEST PERFORMANCE

Previous research showed that the anti-amnestic effect of increased training strength, such as occurred at the higher shock level in Experiment 1, could be blocked by increasing the duration of protein synthesis inhibition [25] The purpose of Experiment 2 was to compare the amnestic effectiveness of a series of three injections of each CAI with a series of three injections of ANI using the passive avoidance task To block the amnestic effect of one or two successive injections of ANI, the footshock was set at 0 36 mA AMPT (160 mg/kg), DDC (250 mg/kg) or TB (10 mg/kg) was administered 1 hr prior to training and at 2-hr intervals thereafter ANI (20 mg/kg) was administered 15 min prior to training and at 2 hr intervals thereafter Control mice received injections of saline using the same injection schedule as for ANI-injected mice The N per group was 20 The retention test was given 1 week after training

With three successive injections of ANI (ANI+ANI+ANI), 90% of the mice were classed as forgetting compared with 10% forgetting for the controls ($p<0$ 01) ANI+ANI+ANI also produced a significantly greater percentage of mice classed as forgetting compared to those mice receiving three successive injections of AMPT, DDC or TB (30%, 10% and 10% respectively) None of the CAIs resulted in a significantly greater percentage of forgetting than the saline control Thus, at this higher level of passive avoidance training, multiple injections of CAIs did not have the same amnestic effect on retention test performance as multiple injections of ANI

EXPERIMENT 3 AMNESTIC EFFECT AND TIME OF DRUG ADMINISTRATION

A single injection of ANI inhibits protein synthesis by 80% or more for 2 hr Since the amnestic effect of ANI is generally believed to be related to the duration of inhibition of protein synthesis following training [25], then one would predict that the amnestic 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 test performance, using the passive avoidance task AMPT, DDC, or ANI was administered at the same dose as in Experiment 2 at 2, 1 or 0 25 hr prior to training The footshock level was 0 34 mA The N per group was 14–20 Retention was tested one week after training

The time of an injection per se had no effect on retention since the saline control group showed 20–25% forgetting across the injection times (Table 8) ANI yielded an amnestic gradient with significantly greater forgetting with ANI administration 0 25 hr rather than 2 hr prior to training ($p<0$ 05) The group receiving ANI 2 hr prior to training did not differ significantly from the control, DDC administered 2 hr prior to training did not differ significantly from the control DDC yielded a nearly constant percent forgetting over the time period AMPT was clearly more effective as an amnestic agent when administered 2 hr prior to training than 0 25 hr prior to training ($p<0$ 05) The results in Table 8 demonstrate that the three agents differed sharply in the temporal course of their amnestic effectiveness

EXPERIMENT 4 EFFECT OF SUBSTITUTION OF CAI FOR ANI

In Experiments 1 and 2 and in previous reports [23,25], it was found that a series of three ANI injections given at 2 hr intervals increased the amnestic effect If the second injection were omitted, then the amnestic effect was absent [22], since the omission allowed recovery of protein synthesis during the memory consolidation period If the mode of action of a PSI as an amnestic treatment is a result of its inhibition of CA synthesis, then one should be able to replace an injection of ANI with an injection of a CAI and still obtain a high percentage of mice classed as forgetting in the PAR task

The mice were trained on PAR So that a comparison of the effectiveness of three injections of ANI+ANI+ANI with ANI+CAI+ANI could be made, the footshock level was set

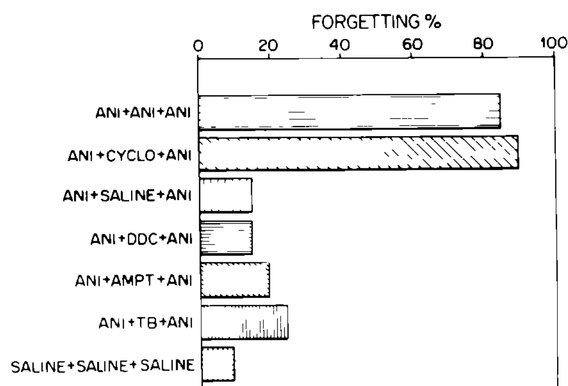

FIG 3 The effect of substituting cycloheximide or catecholamine inhibitors for ANI on memory for the one-trial passive avoidance task The figure shows that three successive injections of ANI yielded a high percent of mice classed as forgetting If saline were substituted for the second ANI injection, the amnestic effect was lost due to recovery of protein synthesis CYCLO could be substituted for the second injection of ANI, and yield a high percentage of mice classed as forgetting DDC, AMPT or TB, substituted for the second injection of ANI, did not give an amnesic effect The N per group was 20

TABLE 9

EFFECT OF THE COMBINATION OF D-AMPHETAMINE WITH AMPT OR ANI

Drug Treatment	Forgetting (%)
Saline (Saline)	10
AMPT (Saline)	25
Saline (d-Amphetamine)	0
AMPT (d-Amphetamine)	90
Saline (Saline)+Saline+Saline	20
ANI (Saline)+ANI+ANI	80
ANI (d-Amphetamine)+ANI+ANI	20
Saline (d-Amphetamine)+Saline+Saline	0

Mice were trained on the T-maze active avoidance task with a footshock intensity of 0 33 mA Because of the differing times required to exert known pharmacological effects, ANI and AMPT were administered under different injection schedules AMPT (160 mg/kg) and its corresponding saline control were administered subcutaneously 1 hr prior to training The three successive injections of ANI required to cause amnesia and the corresponding saline control injections were given 0 25 hr prior to and 1 75 and 3 75 hr after training D-Amphetamine or saline marked in parentheses was administered intraperitoneally immediately after training The N per group was 20 Retention was tested one week after training

at 0 36 mA, as in Experiment 2, so that neither one or two injections of ANI would not cause amnesia All mice received 3 injections The first injection was given 15 min prior to training and was either ANI or saline The second injection was given either 1 hr after the first injection (DDC, AMPT, or TB) to give the inhibitors time to reduce CA levels in brain tissue or 2 hr later (ANI, CYCLO, or saline) The third injection of ANI was given 4 hr after the first injection This resulted in the following groups ANI+ANI+ANI, ANI+CYCLO+ANI, ANI+SALINE+ANI, ANI+DDC-+ANI, ANI+AMPT+ANI, ANI+TB+ANI One group received three successive injections of saline at 2 hr intervals starting 15 min prior to training (SALINE+SALINE +SALINE) to control for the effect of the multiple injections per se on retention test performance

If PSIs impair retention test performance by a mechanism similar to that of CAIs, then ANI+DDC+ANI, ANI+AMPT+ANI and ANI+TB+ANI should have as high a percentage of mice classed as forgetting as ANI+ANI+ANI and ANI+CYCLO+ANI and all groups should have significantly higher percentages of mice classed as forgetting than the ANI+SALINE+ANI On the other hand, if the mechanisms of amnestic action differ, we would predict that substitution of a CAI for a PSI would not lead to amnesia

The percent mice classed as forgetting did not differ significantly between ANI+SALINE+ANI and SALINE+SALINE+SALINE (15% and 10%) Both ANI+ANI+ANI and ANI+CYCLO+ANI yielded a high percentage of mice classed as forgetting (85% and 90%) Both groups differed at $p<0\ 001$ (Chi Square Test) from ANI+SALINE+ANI Substituting DDC, AMPT or TB for the second ANI injection did not result in a high percentage of mice classed as forgetting (15%, 20%, 25% respectively, Fig 3) In fact, none of the ANI+CAI+ANI groups differed significantly from ANI+SALINE+ANI Thus, while CYCLO, an inhibitor of protein synthesis, could be substituted for ANI and result in a high percentage of forgetting, the catecholamine inhibitors, DDC, AMPT or TB, could not

EXPERIMENT 5 ANTI-AMNESIC EFFECT OF D-AMPHETAMINE

D-amphetamine and other stimulants block the amnestic effect of protein synthesis inhibitors [26,28] The purpose of this experiment was to determine if d-amphetamine had the same anti-amnestic effect against AMPT-induced amnesia as against ANI-induced amnesia, using an active avoidance task AMPT was chosen because it blocks both dopamine and norepinephrine synthesis and d-amphetamine is believed to act primarily on dopaminergic synapses [13, 37, 48]

Mice were trained on the T-maze active avoidance task with a footshock intensity of 0 33 mA Because of the differing times required to exert known pharmacological effects, ANI and AMPT were administered under different injection schedules AMPT (160 mg/kg) or saline for its corresponding control was injected 1 hr prior to training The three successive injections of ANI (each 20 mg/kg) required to cause amnesia and the corresponding saline control injections were given 0 25 hr prior to training and 1 75 and 3 75 hr after training D-amphetamine or its saline control was injected immediately after training The second post-training injection is indicated by placing parentheses Retention was tested 1 week after training

Both ANI and AMPT as well as the combinations of ANI + d-amphetamine and AMPT + d-amphetamine given alone had differential effects on retention test performance (Table 9) AMPT had no significant effect on retention compared to the saline injection control, Saline(Saline) Three successive injections of ANI resulted in 80% of the mice being classed as forgetting original training, this differed significantly

TABLE 10

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

Treatment Group	N	Forgetting (%)
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

Mice were trained on the passive avoidance task The footshock intensity was 0 40 mA in order to provide strong training, the latency-to-enter and escape was within the range of 1 0 to 3 0 sec ANI was administered 0 25 hr prior to training and 1 75 hr after training DDC, AMPT, or saline were administered 1 hr prior to training and 1 75 hr after training ECS was administered transcorneally 3 hr after training at 8 mA for 0 2 sec at 60 Hz Mice given pseudo-electroconvulsive shock (P-ECS) were handled in the same manner as the ECS mice except no current was delivered

($p<0\ 01$, Chi Square Test) from the control group, Saline(Saline)+Saline+Saline (20% forgetting) D-amphetamine blocked the amnestic effect of ANI (20% forgetting) but caused amnesia (90% forgetting) when administered to mice that received AMPT Both the differential effects of the ANI and AMPT given alone and in combination with d-amphetamine suggest different mechanisms of action on memory processing

EXPERIMENT 6 INTERACTION OF INHIBITORS WITH ELECTROCONVULSIVE SHOCK

CYCLO and ANI can greatly lengthen the gradient for retrograde amnesia caused by electroconvulsive shock (ECS) ECS given alone was an effective amnestic treatment for passive avoidance training up to 30 min but not 60 min after training The administration of CYCLO prior to training extended the length of the ECS gradient to at least 3 hr [21] Using 1, 2, or 3 successive injections of ANI at 2 hr intervals, it was shown 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 using passive avoidance

Mice were trained on the passive avoidance task Training was made strong enough to block the amnestic effect of ANI, AMPT or DDC when administered alone This was accomplished by increasing the footshock intensity to 0 40 mA All mice received two injections ANI was administered 0 25 hr prior to training and 1 75 hr after training DDC, AMPT, or saline was administered 1 hr prior to training and 1 75 hr after training ECS was administered transcorneally 3 hr after training at 8 mA for 0.2 sec at 60 Hz Only a few mice

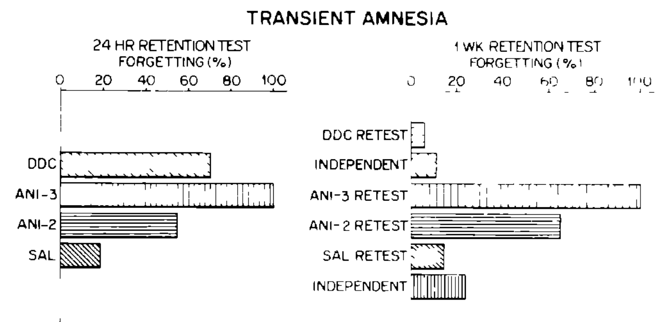

FIG 4 Persistence of amnesia induced by ANI and DDC Mice were administered DDC or ANI prior to passive avoidance training ANI-2 and ANI-3 groups received one or two additional injections 1 75 or 3 75 hr after training Retention was tested 24 hr or 1 week (independent), or both 24 hr and 1 week (retest), after training DDC, ANI-2 and ANI-3 resulted in a significantly greater number of mice being classed as forgetting at 24 hr At the 1 week retest, the ANI injected groups forgot, whereas the DDC groups were not amnestic irrespective of whether retested or independently tested

(<5%) were discarded for failing to show both tonic and clonic convulsions, and several mice died as the result of ECS administration No respiratory assistance was given to mice receiving ECS Mice given pseudo-electroconvulsive shock (P-ECS) were handled in the same manner as ECS treated mice except no current was delivered Retention was tested 1 week after training

As shown in Table 10, the ANI+ANI/P-ECS group was not amnestic (14% forgetting) compared to the control, SALINE+SALINE/P-ECS (15% forgetting) Therefore, any amnestic effect of ANI and ECS treatment could not be due to administration of the protein synthesis inhibitor alone SALINE+SALINE/ECS did not result in amnesia showing that ECS given alone 3 hr after training does not cause amnesia But when ANI and ECS were combined, 92% of the mice were classed as amnesic (ANI+ANI/ECS vs SALINE+SALINE/ECS, Chi Square Test $p<0\ 001$) Neither the DDC+DDC/ECS nor the AMPT+AMPT/ECS treatments significantly lengthened the gradient of ECS retrograde amnesia However, AMPT+AMPT/ECS was close to the 0 05 level of significance ($p<0\ 10$) As expected from the high level of footshock, none of the drug and P-ECS conditions showed appreciable forgetting

EXPERIMENT 7 PERSISTENCE OF AMNESIC EFFECT

Quartermain *et al* [41, 42, 44] reported that CAIs under certain conditions of training and testing induce a transient amnesia, that is, mice appear to forget training when tested 24 hr after training but show normal recall when retested at a later time The purpose of this experiment was to determine if ANI and DDC demonstrate the same phenomenon of inducing transient amnesia

The mice were trained on the passive avoidance task as in Experiment 2 using a footshock level of 0 36 mA Experiment 2 and pilot data indicated that with footshock levels below 0 36 mA, DDC resulted in amnesia when tested 1 week after training DDC might have induced amnesia at 0 36 mA footshock if the retention test had been given at 24 hr DDC (250 mg/kg) was adminstered 3 hr prior to training

A series of two or three injections of ANI were administered starting 15 min prior to training and at 2 hr intervals thereafter (ANI+ANI, ANI+ANI+ANI). Saline was administered 15 min prior to training. Two groups of DDC injected mice and Saline controls were used. One set was tested 24 hr after training and 1 week later, while the other set was tested only 1 week later, so that we could determine the effect of retesting the first group on retention test performance. It was necessary to run a control for repeated testing as repeated testing of mice can serve as a "reminder" procedure that can improve weak memory [14].

At the 24 hr retention test, DDC, ANI+ANI, and ANI+ANI+ANI had poor retention (72%, 60%, 100% forgetting) compared to the saline control (20% forgetting). Of those mice tested 1 week after training, only ANI+ANI and ANI+ANI+ANI showed poor retention (75%, 100%). A comparison of 1-week independent retention tests for DDC and saline versus retest retention test performance yielded no significant difference (Fig. 4). Thus these groups showed continued amnesia, whereas the DDC groups showed only transient amnesia.

DISCUSSION

The present experiments were designed to test further the hypothesis that PSIs may produce a decrement in long-term memory trace formation by their possible side effects on cerebral tyrosine levels and on the rate of accumulation of newly synthesized catecholamines, rather than by their 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 to allow comparison of the biochemical effects on retention test performance.

Experiment 1 was the only experiment in which the CAIs had effects on retention test performance which were similar to those of ANI. ANI and all the CAIs caused comparable amnesia for passive avoidance training at low footshock intensities and failed to cause amnesia at a high footshock level. The results are in accordance with those reported by Quartermain [44]. Experiment 2 showed that CAIs did not cause amnesia for passive avoidance training, under conditions of increased training strength, even when a series of three injections was given, whereas ANI did. In Experiment 3, the amnestic effect of ANI and the CAIs did not show the same temporal relation between time of pretraining injection and percent forgetting. ANI was more effective when given 15 min rather than 1 or 2 hr prior to training. 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 rather than 15 min prior to training. In Experiment 4, three successive injections of ANI caused amnesia. When the second of three ANI injections was changed to CYCLO, another protein synthesis inhibitor, a high percentage of the mice were amnestic. When the second ANI injection was changed to saline, amnesia did not occur. However, when DDC, AMPT or TB was substituted for the second ANI injection, no significant amnestic effect was observed. In fact, the CAI substitution for ANI had no greater effect on retention than a saline injection administered at the same time. In Experiment 5, ANI caused amnesia but AMPT did not for T-maze training. D-amphetamine blocked the amnesia induced by ANI, but AMPT and d-amphetamine resulted in 90% forgetting. In Experiment 6, a single ECS at 3 hr after passive avoidance training did not cause amnesia. Two successive injections of ANI followed by ECS at 3 hr was highly amnestic. However, two successive injections of neither AMPT nor DDC, followed by ECS at 3 hr, yielded significant amnestic effects. Experiment 7 showed that DDC induced transient amnesia for passive avoidance training when the footshock intensity was high. That is, DDC impaired retention test performance at 24 hr but not at 1 week. The results were the same whether mice were retested at 1 week or independent groups were tested. ANI did not show transient amnesia under these conditions of training, both ANI+ANI and ANI+ANI+ANI resulted in poor retention test performance at 24 hr and when retested 1 week later. A single injection of ANI was not tested since it was already known not to cause amnesia at this footshock level in passive avoidance training at 24 hr or 1 week.

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 CAIs 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 CAIs were not effective amnestic agents in paradigms in which protein synthesis inhibitors were effective and that CAIs could not be used interchangeably with protein synthesis inhibitors to obtain the same amnestic effects. This also suggests different mechanisms of action on memory formation for protein and catecholamine synthesis inhibitors. In addition, the anatomical substrate differs for AMPT and DDC versus CYCLO and ANI [30]. If the mechanism of action were similar, then one would expect to find far greater similarity in their biochemical and behavioral effects.

We do not wish to suggest that neurotransmitters are not important for long-term memory formation, since evidence presented by ourselves and others indicates that interference with synaptic transmission of DA, NE, 5-HT, GABA, glutamic acid, glycine and ACh can alter memory processing. We have found that while AMPT and DDC are only marginally effective amnestic agents when given subcutaneously, they are highly effective when administered intracerebrally into specific areas of the brain [30]. The available evidence is still consistent with the hypothesis that synthesis of brain proteins is required for long-term memory trace formation, and that ANI, and most likely CYCLO and acetoxycycloheximide, are effective amnestic agents due to their action as inhibitors of protein synthesis [46,47].

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