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EFFECT OF CHOLINE ACETYLTRANSFERASE INHIBITORS ON MOUSE AND GUINEA-PIG BRAIN CHOLINE AND ACETYLCHOLINE*

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Abstract—These experiments measure the effect of two choline acetyltransferase (CAT) inhibitors, viz. 4-(1-naphthylvinyl) pyridine (NVP) and 4-(3-chlorophenylvinyl) pyridine (3′-chloro-4-stilbazole; CS), on mouse and guinea-pig brain acetylcholine (ACh) and choline. The intraperitoneal administration of NVP or CS appeared to inhibit CAT partially in both species, but both compounds were without effect on steady-state levels of ACh. In the mouse CS, but not NVP, increased brain choline. Both CS and NVP were shown to reduce, but not totally inhibit, the synthesis of mouse brain ACh. The inability of these compounds to decrease steady state levels, while apparently decreasing synthesis rates, suggests that CAT may not be the rate-limiting step in ACh synthesis.

Acetylcholine (ACh) is synthesized from the substrates, choline and acetyl-CoA, via the enzyme, choline acetyltransferase (CAT: acetyl CoA: choline *O*-acetyltransferase; EC 2.3.1.6.). A number of styrylpyridine analogues have been synthesized which have been shown to be potent inhibitors of this enzyme *in vitro* [1–3]. Recent studies have indicated that these compounds appear to inhibit partially brain CAT after administration *in vivo* [4], but do not decrease steady state levels of brain ACh [5]. However, these investigations did not provide conclusive evidence that CAT inhibitors reach the enzyme *in vivo*.

This investigation provides further information on the neurochemical effects of two styrylpyridine analogues, viz. 4-(1-naphthylvinyl) pyridine (NVP) and 4-(3-chlorophenylvinyl) pyridine (3′-chloro-4-stilbazole; CS).

METHODS AND MATERIALS

General procedures. Male (CF-1) albino mice and male guinea-pigs (Carworth Farms, Becton, N.Y.) were used. Mice (17–31 g) and guinea-pigs (300–600 g) were injected intraperitoneally with drug solutions or appropriate vehicles in a volume of 10 ml/kg.

Animals were killed by cervical dislocation and immediately taken into a cold room (4°) where the brain (minus cerebellum, pons and medulla) was removed, sectioned through the central sulcus, and each half weighed. One-half of the brain was homogenized (0–4°) in 1 N formic acid–acetone (15:85, v/v; 150 mg/ml for ACh and choline assays); the contralateral half was homogenized (0–4°) in distilled water (CAT assays). Formic acid–acetone homogenates were allowed to stand for 30–60 min in the cold (4°) to allow for the complete extraction of ACh [6]. For ACh and choline determinations in mice, the time interval between death and homogenization was less than 1 min; for guinea-pigs, the interval was about 2 min. Unless otherwise indicated, all homogenizations were performed in ground-glass homogenizers.

Assays. Choline and ACh were assayed in duplicate by the radio-enzymatic assay of Goldberg and McCaman [7, 8]. Mouse and guinea-pig brain CAT was assayed by the method of McCaman and Hunt [9] as modified by Spyker *et al.* [10]. Mouse adrenal glands were prepared and assayed for CAT [11].

During the course of these experiments, it was noted that mouse brain CAT activity, but not guinea-pig CAT activity, varied between 65–100 μmoles/g of protein/hr. This type of variation was never found to occur on any one day but rather between days. The per cent inhibition of total enzyme activity produced by a single maximally effective dose of either NVP or CS was independent of the observed daily variation in CAT activity. Thus, data obtained from experiments repeated on different days, which demonstrated such variability, are expressed as a per cent of concurrently run controls rather than as absolute values. A similar phenomenon was observed with mouse brain ACh (14–28 nmoles/g wet wt) and choline levels (60–120 nmoles/g wet wt) and the data were analyzed as described above for CAT activity. Control animals were always assayed simultaneously with treated animals. Animals used in this study were killed between 0900 and 1400 hr.

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Subcellular distribution. Approximately 300 mg mouse brain was homogenized in 6·75 ml of ice-cold 0·32 M sucrose in a glass homogenizer fitted with a motor-driven Teflon pestle with a clearance of 0·025 cm [12]. An aliquot of the homogenate was removed for analysis of CAT, while the remainder of the homogenate was centrifuged at 1000 *g* for 10 min. The resulting pellet (P_1, containing unbroken cells and debris) was resuspended in 3 ml of 0·32 M sucrose and recentrifuged as above. The supernatants from the two centrifugations were combined and centrifuged at 12,000 *g* for 20 min and the P_2 fraction obtained (crude mitochondrial pellet) was resuspended in 3 ml sucrose and recentrifuged as above. The two supernatant fractions (S) were pooled and analyzed for CAT. The homogenate and the P_1 and P_2 pellets were resuspended in water (9 ml H_2O/g of original tissue), rehomogenized in ground-glass homogenizers and assayed for CAT. Under these conditions, the distribution of CAT was similar to that reported by McCaman *et al.* [13]. All phases of the fractionation and centrifugation procedure were carried out at 0–4°. Each fraction was assayed for protein [14].

Stress experiments. To assess the effect of a stress, mice were pretreated with saline or 250 mg/kg of NVP; 80 min after injection, the animals were forced to swim for 10 min in water at room temperature (22°). After the swim period, they were removed from the tank, immediately killed, and the brain was removed and analyzed for ACh and CAT activity. Two groups of saline-injected controls were used: one group was forced to swim for 10 min, while the second group did not. There was no difference in either brain ACh or CAT activity between the two control groups.

Drugs and solutions. Solutions of 4-(1-naphthylvinyl) pyridine hydrochloride and 4-(3-chlorophenylvinyl) pyridine hydrochloride were prepared immediately before use. NVP was dissolved in an appropriate volume of 0·9% NaCl, whereas CS was dissolved in a 1:1 (v/v) mixture of propylene glycol–0·9% saline. Doses refer to the salt form and, unless otherwise specified, the *trans*-isomers of both CS and NVP were used.

Solutions of NVP are light-sensitive, being converted from the active *trans*-isomer to a mixture of *trans*- and the less active *cis*-forms (hereafter referred to as *cis*-NVP) with a predominance of the *cis*-isomer [15]. When the *cis*-isomer of NVP was used, a freshly prepared solution of the *trans*-isomer was allowed to stand in direct sunlight for at least 90 min so that the maximal formation of the *cis*-form occurred [15]. When it was necessary to carry out any part of an experiment in the absence of normal room light, a room lighted by a single 25-W red bulb was used.

RESULTS

Inhibition in vitro *of choline acetyltransferase by isomers of NVP.* Both isomers of NVP inhibited CAT activity in mouse brain homogenates in a concentration-dependent manner. The I_{50} (concentration inhibiting 50 per cent of the enzyme activity) for *trans*-NVP was $9{\cdot}4 \times 10^{-7}$ M. The I_{50} for *cis*-NVP was $1{\cdot}2 \times 10^{-5}$ M; however, it must be emphasized that the *cis*-isomer was formed from solutions of *trans*-NVP by photoisomerization and contained significant amounts of the unchanged *trans*-form. Hence, a major portion, if not all, of the apparent activity of *cis*-NVP is probably due to contaminating *trans*-NVP. The very limited water solubility of CS precluded an analysis of this compound *in vitro.*

Inhibition in vivo *of choline acetyltransferase by NVP and CS.* The administration of either CS or NVP *in vivo* produced a dose-dependent decrease in measureable enzyme activity (Table 1). The maximal inhibition observed with either compound was approximately 60 per cent.

Mice injected with 250 mg/kg of CS demonstrated a significant ($P < 0{\cdot}05$) enzyme inhibition 17 hr later (50·8 ± 2·8 vs. 82·8 ± 4·2 μmoles/g protein/hr, CS and controls respectively). On the other hand, mice injected with 250 mg/kg of NVP demonstrated no enzyme inhibition at this time period (81·9 ± 1·6 μmoles/g protein/hr).

Effect of administration of CAT inhibitors in vivo *on brain acetylcholine and choline content.* No decrease in

Table 1. Inhibition of mouse brain CAT activity *in vivo* by NVP and CS*

	NVP			CS		
Dose (mg/kg)	n†	CAT activity (μmoles/g protein/hr)	% of Control	n†	CAT activity (μmoles/g protein/hr)	% of Control
0	3	75·2 ± 1·1‡	100	3	70·8 ± 1·2	100
50	3	47·1 ± 2·6	62·7 ± 3·5	3	48·3 ± 2·1	68·2 ± 3·0
100	3	38·7 ± 5·1	51·5 ± 6·8	3	39·5 ± 0·8	55·7 ± 1·2
200	3	28·4 ± 1·2	37·7 ± 1·5	3	28·7 ± 1·8	40·6 ± 2·6
400	3	23·4 ± 1·8	31·0 ± 2·4	3	27·0 ± 5·4	38·2 ± 7·7

* Mice were injected with varying doses of NVP or CS, killed 30 min later, and the brain was assayed for CAT activity.
† Number of animals; each brain was assayed in triplicate.
‡ Values are means ± S.E.M.

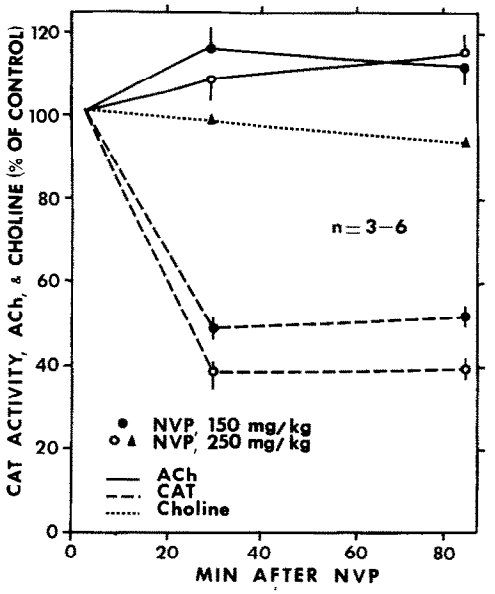

Fig. 1. Effect of NVP on mouse brain CAT activity, choline and ACh levels. Mice were injected with 150 or 250 mg/kg of NVP and killed either 30 or 90 min later. One-half of the brain was assayed for CAT activity; the contralateral half was assayed for ACh. Choline was determined in a separate series of experiments. CAT activity, choline and ACh levels are expressed as a per cent of concurrently run controls. n = Number of animals; vertical lines are S.E.M. Control levels of CAT, ACh and choline (±S.E.M.) were: 76·5 ± 2·4 μmoles/g protein/hr and 17·5 ± 0·6 nmoles/g and 114 ± 4·4 nmoles/g respectively.

brain ACh levels was apparent either 30 or 90 min after administration of NVP in doses of 150 or 250 mg/kg (Fig. 1). Indeed, ACh levels were significantly elevated ($P < 0.05$) at the 30-min time period after the smaller dose of NVP. CAT activity was apparently decreased by approximately 60 per cent throughout the 90-min period. Brain choline was not affected by the higher dose of NVP (Fig. 1).

Doses of CS that were apparently maximally effective, i.e. 250 mg/kg, were also without effect on brain

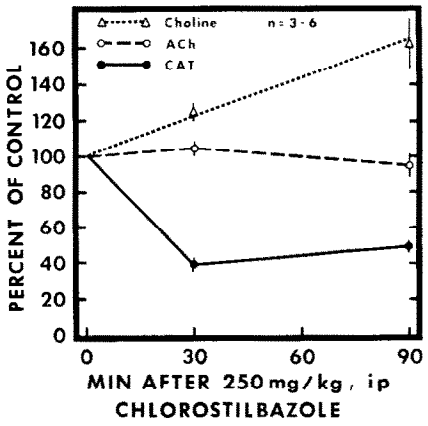

Fig. 2. Effect of CS on brain CAT, ACh and choline. Mice treated with 250 mg/kg of CS were killed either 30 or 90 min after injection. One-half of the brain was assayed for CAT; the contralateral half was assayed for ACh and choline. n = Number of animals at each point; vertical lines are S.E.M. Control levels of CAT, ACh and choline (±S.E.M) were: 70·4 ± 1·8 μmoles/g protein/hr, 25·7 ± 1·3 nmoles/g and 64·1 ± 3·3 nmoles/g respectively.

ACh levels (Fig. 2). As with NVP, CAT activity appeared to be reduced by about 60 per cent during the 90-min period examined. As opposed to NVP, brain choline levels were significantly elevated at both the 30- and 90-min time periods after CS. The early increase in brain choline levels was somewhat variable from experiment to experiment (cf. Fig. 2 and Fig. 4); however, an increase was always apparent at longer time periods.

In a further experiment, mice received two injections of NVP (250 mg/kg) administered 8 hr apart and were killed 60 min after the second injection. CAT was inhibited by 70 per cent; however, no decrease in brain ACh was evident (18·5 ± 0·8 and 17·4 ± 0·4 nmoles/g for NVP-treated and controls respectively).

NVP and CS are both CNS depressants. NVP (250 mg/kg) produces a marked decrease in spontaneous motor activity. CS, at the same dose, produced

Table 2. Subcellular distribution of CAT in control and NVP-treated mouse brain*

Fractions	Treatment			
	Control (μmoles/g protein/hr)	NVP (μmoles/g protein/hr)	% of Control	P
Whole homogenate	64·8 ± 0·8†	34·6 ± 1·9	53·4 ± 2·9	<0·05
Nuclear pellet (P_1)	36·3 ± 1·1	25·3 ± 2·3	69·6 ± 6·3	<0·05
Crude mitochondrial pellet (P_2)	108 ± 1·2	42·5 ± 1·2	39·3 ± 1·1	<0·05
Supernatant	43·5 ± 3·3	20·0 ± 0·8	46·1 ± 2·0	<0·05

* Mice were treated with saline or 250 mg/kg of NVP and killed 120 min later. Brains were removed and homogenized in 0·32 M sucrose and subjected to differential centrifugation as described in Methods. There were three animals in the control group and four in the NVP-treated group.

† Values are means ± S.E.M.

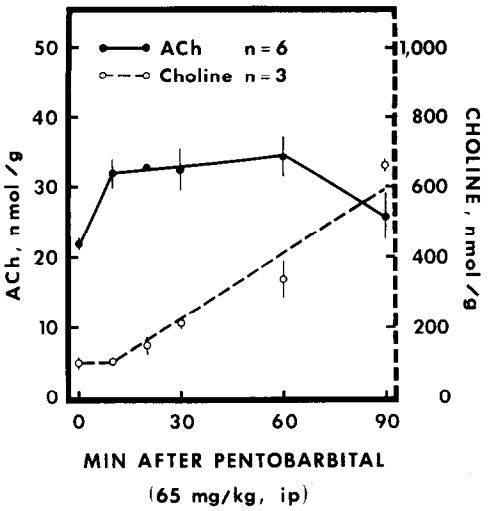

Fig. 3. Effect of pentobarbital on mouse brain ACh and choline. Animals were treated with 65 mg/kg of pentobarbital, killed at varying time intervals after injection, and the brain was analyzed for ACh and choline. n = Number of animals at each point; vertical lines are S.E.M. Where standard errors are omitted, they were smaller than the symbol for the mean.

a loss of the righting reflex in approximately 50 per cent of the animals.

Experiments demonstrating inhibitor effectiveness against mouse brain CAT in vivo. Preparation of subcellular fractions from brains of mice pretreated with NVP demonstrated that "synaptosomal" CAT was inhibited by approximately 60 per cent (Table 2). The nuclear pellet and the supernatant fraction also demonstrated significant levels of enzyme inhibition. There was no observable enzyme inhibition in any of the subcellular fractions when control brains were homogenized in iso-osmotic sucrose containing 2×10^{-5} M NVP.

Since the above results could conceivably be explained by inhibitor binding, largely to external membrane sites, and a redistribution of the inhibitor during the homogenization of synaptosomes, a second approach was to examine the effect of inhibitor pretreatment on drug-induced elevations in brain ACh.

Pentobarbital is well known to increase steady state levels of brain ACh. Figure 3 illustrates the time course of the effect of pentobarbital on mouse brain choline and ACh. Ten min after pentobarbital treatment, brain ACh reached a new steady state and remained elevated for about 1 hr. The time course of changes in brain choline levels after pentobarbital differed markedly from ACh. During the initial 10-min period, choline levels did not change. However, at the 10-min period, when brain ACh reached a new steady state, choline began to rise in a relatively linear fashion for the next 80 min. Experiments with pentobarbital at shorter time periods, i.e. 0–10 min, demonstrated that 10 min was the time of peak increase in brain ACh.

The effect of CS pretreatment on pentobarbital-induced increases in brain ACh and choline is illustrated in Fig. 4. Figure 4A demonstrates that pentobarbital did not interfere with CS-induced inhibition of brain CAT. Figure 4B illustrates that, 40-min after CS administration, brain ACh levels were significantly ($P < 0.05$) increased compared to controls, whereas no increase was apparent at the 60-min period. Ten and 30 min after pentobarbital alone, steady state levels of brain ACh were significantly elevated ($P < 0.05$). Mice treated with CS 30 min prior to pentobarbital and killed 10 min after pentobarbital, i.e. at the pentobarbital peak, demonstrated brain levels of ACh lower than those of animals receiving pentobarbital alone. Figure

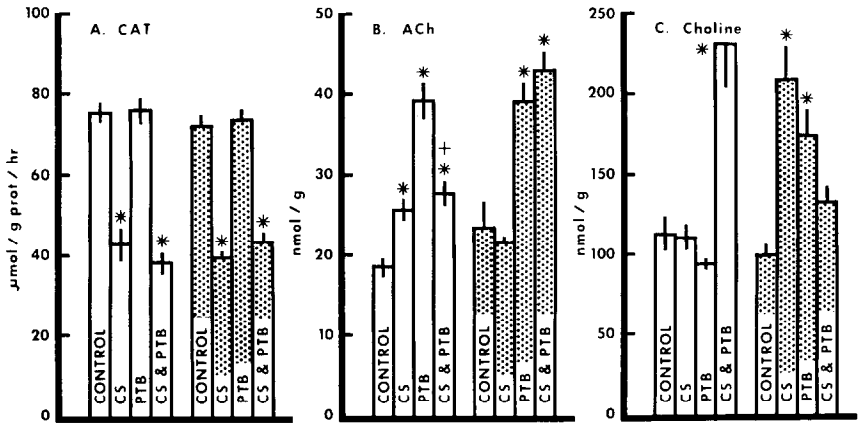

Fig. 4. Effect of CS pretreatment on pentobarbital-induced increases in brain ACh and choline. Animals were treated with 250 mg/kg of CS followed 30 min later by pentobarbital (65 mg/kg). Animals were killed either 10 or 30 min after pentobarbital and the brain was analyzed for CAT, ACh and choline. Open columns represent the 10-min period; stippled columns represent the 30-min period after pentobarbital. Each bar represents the mean ± S.E.M. of four animals. The symbol (*) = $P < 0.05$ when compared to corresponding controls; (+) = $P < 0.05$ when compared to pentobarbital alone.

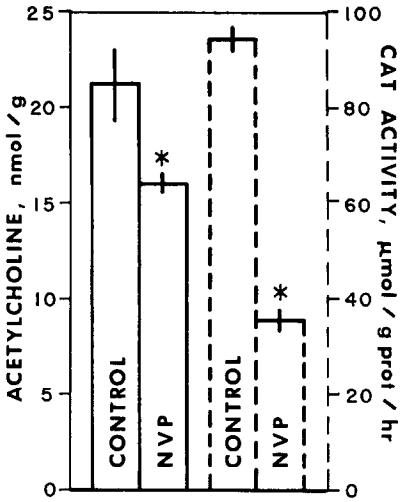

Fig. 5. Effect of a swim stress on brain ACh after NVP. Mice were treated with 250 mg/kg of NVP; 80 min later they were forced to swim for 10 min in water at room temperature (22°). After swimming, they were killed and the brain was analyzed for CAT and ACh. One group of controls did not swim; however, brain ACh and CAT levels of swimming versus nonswimming controls did not differ; hence, they were combined. Each bar represents the mean ± S.E.M. of three to six animals. The asterisk = $P < 0.05$.

4C illustrates that mice treated with CS or pentobarbital 40 or 10 min, respectively, prior to killing demonstrated no significant change in brain choline levels compared to controls. However, the combination of CS and pentobarbital, neither of which affected brain choline by itself, more than doubled choline levels ($P < 0.05$). Mice killed 60 min after CS or 30 min after pentobarbital administration demonstrated significant ($P < 0.05$) elevations in brain choline.

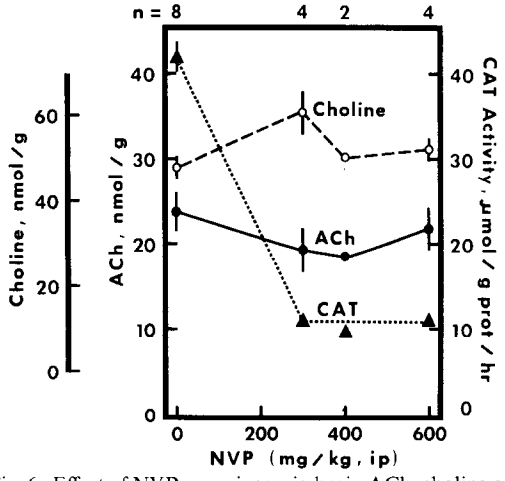

Fig. 6. Effect of NVP on guinea-pig brain ACh, choline and CAT. Guinea-pigs were injected with varying doses of NVP and killed 60 min later. Brains were analyzed for CAT, ACh and choline. n = Number of animals at each point; vertical lines are S.E.M.

A third approach to illustrate the effectiveness of these compounds *in vivo* was to determine the effect of a stress on brain ACh after administration of a maximally effective dose of NVP. Animals were treated with 250 mg/kg of NVP and 80 min later were forced to swim for 10 min. Figure 5 illustrates that NVP-treated mice demonstrated a significant ($P < 0.05$) decrease (20 per cent) in brain ACh as compared to controls. CAT appeared to be inhibited by about 60 per cent ($P < 0.05$).

Effect of NVP on guinea-pig brain CAT activity, ACh and choline. Figure 6 illustrates that the apparent maximal obtainable level of CAT inhibition after 300 mg/kg of NVP in the guinea-pig was about 75 per cent, somewhat greater than that seen in the mouse. Increasing the dose to 400 or 600 mg/kg produced no additional enzyme inhibition. Figure 6 also illustrates that, as seen in the mouse, neither brain choline nor brain ACh was significantly affected by the enzyme inhibition. At all three doses used, depression was apparent; however, it was not as marked as as that in the mouse.

Effect of NVP on mouse adrenal CAT activity. Mice treated with 250 mg/kg of NVP were killed 90 min later and adrenal pairs analyzed for CAT activity. A significant ($P < 0.05$) 38 per cent decrease in enzyme activity was apparent (21.5 ± 1.7 and 13.2 ± 0.9 nmoles/adrenal pair/hr, for controls and NVP-treated animals respectively). This level of enzyme inhibition is lower than that found in the whole brain (cf. Table 1).

DISCUSSION

The administration *in vivo* of two potent *in vitro* styrylpyridine CAT inhibitors, NVP and CS, to mice or guinea-pigs did not decrease steady state levels of brain ACh in either species. The results of this investigation are in agreement with studies by Ross *et al.* [16], who used a choline haloacetate type of inhibitor, and the studies of Carson *et al.* [5], using styrylpyridine derivatives. Furthermore, Saelens (personal communication) studied effects *in vivo* of a variety of *in vitro* CAT inhibitors and has found none capable of decreasing brain ACh content.

Theoretically, administration of CAT inhibitors *in vivo* should produce a decrease in brain ACh content. It is reasonable to propose, therefore, that these compounds may be ineffective *in vivo* for a number of reasons: (1) rapid inactivation of the compounds *in vivo*, (2) inability of the inhibitors to penetrate the blood–brain barrier, (3) confinement to extracellular, non-neuronal or non-cholinergic tissues in the brain, or (4) CAT may not be the rate-limiting step in ACh synthesis.

The experiments reported here, however, provide evidence that these compounds do reach brain CAT in a pharmacologically active form and, to a degree, inhibit the enzyme's activity. This conclusion is supported by the following evidence: First, subcellular

fractions prepared from the brains of mice treated with NVP demonstrated significant levels of enzyme inhibition in all fractions. These results suggest that the inhibitors are capable of penetrating the blood–brain barrier as well as neuronal membranes in a pharmacologically active form. Second, the rate of rise of brain ACh induced by pentobarbital is significantly reduced by prior treatment with an inhibitor, indicating a decreased synthetic capability. Third, mice subjected to a swim stress, after treatment with an inhibitor, demonstrated significant decreases in brain ACh content. On the basis of these experiments, it appears highly probable that CAT is not the rate-limiting step in the synthesis of mouse brain ACh.

Throughout this study, brains from mice treated with the enzyme inhibitors were removed and analyzed *in vitro* for CAT activity. When dealing with irreversible enzyme inhibitors, this is an acceptable procedure; however, the irreversibility of styrylpyridine inhibitors has not been established. Indeed, unpublished experiments in our laboratory, as well as observations by Cavallito [17], suggest that these compounds may not be irreversible inhibitors. Therefore, the 60 per cent level of enzyme inhibition measured in these studies cannot be regarded as a reflection of the true level of enzyme inhibition.

Styrylpyridine CAT inhibitors possess a variety of undesirable pharmacological effects and, while they are apparently partially effective against brain CAT, they are unable to inhibit the enzyme completely. On this basis, it would appear that these compounds are pharmacologically ineffective inhibitors; however, their inability to reduce steady state levels of ACh may be related to the cholinergic nervous system and not to the efficacy of these agents as enzyme inhibitors.

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REFERENCES

1. J. C. Smith, C. J. Cavallito and F. F. Foldes, *Biochem. Pharmac.* **16,** 2438 (1967).
2. C. J. Cavallito, H. S. Yun, J. C. Smith and F. F. Foldes, *J. med. Chem.* **12,** 134 (1969).
3. B. R. Baker and R. E. Gibson, *J. med. Chem.* **14,** 315 (1971).
4. M. E. Goldberg, A. I. Salama and S. W. Blum, *J. Pharm. Pharmac.* **23,** 384 (1971).
5. V. G. Carson, D. J. Jenden and A. K. Cho, *Proc. west. Pharm. Soc.* **15,** 127 (1972).
6. M. Toru and M. H. Aprison, *J. Neurochem.* **13,** 1533 (1966).
7. A. M. Goldberg and R. E. McCaman, *J. Neurochem.* **20,** 1 (1973).
8. A. M. Goldberg and R. E. McCaman, in *Choline and Acetylcholine: Handbook of Chemical Assay Methods* (Ed. I. Hanin), p. 47. Raven Press, New York (1974).
9. R. E. McCaman and J. M. Hunt, *J. Neurochem.* **12,** 253 (1965).
10. J. M. Spyker, S. B. Sparber and A. M. Goldberg, *Science, N.Y.* **177,** 621 (1972).
11. A. M. Goldberg and B. L. Welch, *Science, N.Y.* **178,** 319 (1972).
12. E. G. Gray and V. P. Whittaker, *J. Anat.* **96,** 79 (1962).
13. R. E. McCaman, G. R. DeLores Arnaiz and E. DeRobertis, *J. Neurochem.* **12,** 927 (1965).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
15. H. L. White and C. J. Cavallito, *Biochim. biophy. Acta* **206,** 242 (1970).
16. S. B. Ross, L. Florvall and O. Frödén, *Acta pharmac. toxic.* **30,** 396 (1971).
17. C. J. Cavallito, in *Interaction of Chemical Agents with Cholinergic Mechanisms*, Biological Conference "Oholo," p. 28. Israel Inst. Biol. Res. Ness-Ziona (1971).