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EFFECTS OF THE CHOLINE ACETYLTRANSFERASE INHIBITOR 3'CHLORO-4-STILBAZOLE ON BRAIN ACETYLCHOLINE METABOLISM

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Abstract—3'Chloro-4-stilbazole (CS), an effective inhibitor *in vitro* of choline acetyltransferase (ChA), was tested *in vivo* in rats and mice. Brain concentrations of CS were measured and were as high as 1 m-mole kg^{-1} 15 min after 0·79 m-mole kg^{-1} of CS was injected i.p. in rats. Its half-life is about 3 hr, yet no changes in total brain acetylcholine (ACh) or choline (Ch) levels were seen after acute injections. However, the rate of synthesis of $^2H_4$-ACh in rat brain after i.v. $^2H_4$-Ch was significantly decreased after doses of 200 or 400 μmoles kg^{-1} of CS. Repeated injections in rats reduced the total ACh level to 87 per cent ($P < 0·05$) of the control level. Atropine sulfate (7·2 μmoles kg^{-1}), alone or in combination with CS, reduced rat brain ACh levels to 65 per cent of normal. In mice, both total brain ACh and Ch levels were moderately but significantly elevated with an acute intraperitoneal injection of CS. ACh turnover was significantly decreased after doses of 200 or 400 μmoles kg^{-1} of CS, yet it was significantly increased after an injection of 40 μmoles kg^{-1} of CS. It is concluded that either ChA is not a rate-limiting enzyme in the biosynthesis of ACh in brain or CS fails to gain access to ChA.

Recently Baker and Gibson [1] described a series of 4-stilbazole analogs which were inhibitors *in vitro* of choline acetyltransferase (ChA). Of the 43 derivatives that were tested, 3'chloro-4-stilbazole (CS) (mol. wt. 252) was reported to be the most selective in that its I_{50} toward ChA (7·8 μM) was 1/130 that for acetylcholinesterase (AChE) (1 mM) under the conditions used for the assays. Substrate concentrations *in vivo* are not known and effects *in vivo* cannot be accurately predicted from these data, but the apparently high selectivity of CS suggested its use in examining the role of ChA in regulating acetylcholine (ACh) levels in the brain.

In this report, we describe the results of experiments intended to examine the effects of CS on brain ACh and choline (Ch) levels and turnover *in vivo*. A chemical estimation procedure was devised and used to measure CS levels in brain and blood so that the concentration dependence of the effects could be assessed. A preliminary account of some of this work has been published [2].

METHODS

Estimation of 3'-chloro-4-stilbazole

Brain. The whole brain was removed, weighed and homogenized in sufficient pH 7·4 phosphate buffer to give a 10% (w/v) solution. Duplicate 2·0-ml aliquots were shaken with 5·0 ml heptane for 10 min in screw-cap test tubes, then centrifuged at 3000 rev/min for 2 min. Three ml of the heptane phase was then extracted with 1·5 ml of 0·1 N HCl by shaking for 10 min. After centrifuging at 3000 for 2 min, the optical density of the HCl phase at 330 nm was measured. The concentration of CS was determined by means of a standard curve prepared by adding known quantities of CS to the HCl. The recovery of CS added to brain homogenates was 87 per cent.

The specificity of the assay was established by comparing the u.v. spectra of the HCl extract and authentic CS in HCl. Metabolic hydroxylation of either of the aromatic rings would cause substantial changes in the u.v. spectrum of CS. There were no significant differences between the spectrum of authentic CS and that of the extract from plasma or tissue (Fig. 1).

Plasma. Blood was collected in heparinized syringes, transferred to heparinized tubes and centrifuged at 4000 rev/min for 20 min. Two-ml aliquots of the plasma were assayed for CS in the same manner as the brain homogenates.

CS half-life in brain and plasma. Seventeen male Sprague–Dawley rats (150–250 g) were given 1 ml/kg

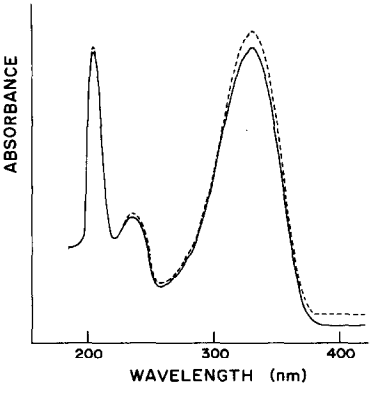

Fig. 1. Ultraviolet absorption spectrum of authentic CS (40 μM) (solid line) and extract of rat brain prepared as described in the text (dotted line) after intraperitoneal administration of CS (790 μmoles kg^{-1}). The ordinate is 2 absorbance units full scale.

i.p. injections of 0·79 M CS. Animals were sacrificed at each of the following times after injection: 0·25, 0·5, 1·0, 1·5, 4, 8 and 24 hr. Mean brain and plasma levels of CS were determined as described above.

Estimation of ACh levels in rats

ACh and Ch levels after various doses of CS. Twenty-eight male Sprague Dawley rats (175–215 g) were divided into groups of at least four each and given i.p. injections of one of the following: 1 ml/kg of the vehicle (30% Solketal in normal saline), 40 μmoles/kg of CS, 100 μmoles/kg of CS, 200 μmoles/kg of CS or 400 μmoles/kg of CS. Twenty min after the i.p. injection, each rat received 20 μmoles/kg of $^2H_4$-Ch i.v. and was sacrificed by decapitation 30 sec later. The brains were rapidly removed, frozen in liquid nitrogen, and analyzed for $^2H_0$- and $^2H_4$-Ch and ACh by GC/MS [3,4].

ACh levels after repeated injections of CS. Twelve rats received CS (790 μmoles kg^{-1} i.p.), followed by two additional doses of 400 μmoles kg^{-1} at 4 and 8 hr after the initial injection. All injections were of 1 ml kg^{-1} of CS solution in 30% propylene glycol in normal saline. Six control animals received 1 ml kg^{-1} of the vehicle i.p. at the same times as the control animals. All animals were sacrificed by decapitation 9 hr after the first injection. Each brain was rapidly removed, frozen in liquid nitrogen and pulverized. The pulverized material from each brain was divided in about half and weighed. One part was used for a CS determination and the second for an ACh determination.

ACh levels after repeated injections of atropine or atropine plus CS. Six rats received atropine sulfate (7·2 μmoles kg^{-1}) in 1 ml kg^{-1} of normal saline at 0, 4 and 8 hr. Six rats received the same i.p. injection of atropine sulfate and i.p. injections of CS (400 μmoles kg^{-1}) at 0, 4 and 8 hr. Four control animals received no injections. Hypothermia was prevented by placing all animals in a 32° incubator at 0 time and keeping them there for 9 hr. They were then sacrificed by decapitation and the brains were treated as described in the previous paragraph.

ACh turnover in mice

ACh turnover rate was estimated using 251 male Swiss–Webster mice (24–30 g) by the method described by Jenden *et al.* [4, 5]. Groups of at least four mice each were injected intravenously with $^2H_4$-Ch (20 μmoles kg^{-1}) 20 min after the intraperitoneal injection of various doses of CS. At intervals of 20 sec, 40 sec, 1 min, 2 min, 4 min, 8 min, 15 min and 30 min after the injection of Ch, the animals were sacrificed by cervical dislocation and the brains were rapidly removed and frozen (20–25 sec) in liquid nitrogen. Brains were analyzed for $^2H_0$- and $^2H_4$-Ch and ACh by GC/MS [4]. The ACh specific activities were plotted serially for each concentration of CS and the slopes of the initial rising part of the curves were calculated. These initial rates of $^2H_4$-ACh increase represent various minimum estimates of the rate of synthesis of ACh [5]. The turnover estimates were corrected for changes in the specific activity of Ch by using the formula $dy^*/dt = V(x^*/x - y^*/y)$, where x and y nmoles g^{-1} denote total concentrations of Ch and ACh, an asterisk denotes a labeled variant and V equals the turnover rate. The empirical equation $y^* = A(e^{-\alpha t} - e^{\beta t})$ was fitted by nonlinear regression analysis to each $^2H_4$-ACh curve and differentiated at each point to estimate dy^*/dt. Then dy^*/dt was plotted against $(x^*/x - y^*/y)$ for each concentration of CS, and the slopes of lines going through the origin were used to estimate the various values of V [5].

RESULTS

Brain and plasma levels of CS

The decline in brain levels of CS with time after a dose of 0·79 m-mole kg^{-1} is shown in Fig. 2. Thirty min after injection, the brain levels of CS were 690 ± 79 (S. E. M.) nmoles g^{-1}, while plasma levels were 51 ± 16 nmoles g^{-1}. This approximately 10-fold concentration difference was maintained 4 and 8 hr after injection. The brain and plasma levels declined with a half-life of about 2·7 hr.

ACh levels after CS

ACh and Ch levels after various doses of CS. In rats, total brain levels of ACh and Ch were not significantly altered 20·5 min after varying doses of CS (Table 1). CS significantly lowered $^2H_4$-ACh levels found in rat brain 30 sec after i.v. $^2H_4$-Ch (4, 20; $F = 7·47$), while the tendency for CS to decrease $^2H_4$-Ch levels was not significant (Fig. 3). The decrease in $^2H_4$-ACh after CS indicates a reduction in the turnover of ACh.

ACh levels after repeated injections of CS. A significant depression of ACh of 13 per cent ($P < 0·05$, $t = 2·484$) was observed with brain levels of CS at 342 nmoles/g (Table 2). There was no significant correlation between ACh levels and CS levels ($r = -0·045$, $n = 12$).

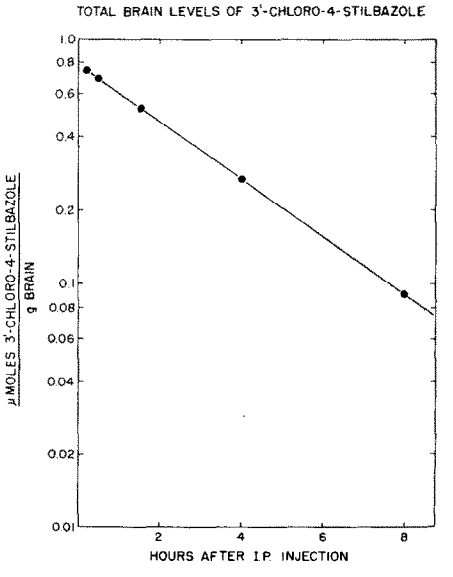

Fig. 2. Mean rat brain levels of CS at various times after intraperitoneal administration of CS (790 μmoles kg^{-1}). Each point represents the average of two or more animals. No CS was detected in the brains of four animals sacrificed 24 hr after the same dose.

Table 1. Brain levels of choline and acetylcholine after various doses of CS

Dose of CS† (μmoles kg^{-1})	Mean brain levels* Acetylcholine (nmoles g^{-1})	Choline (nmoles g^{-1})
0	26·4 ± 0·8 (5)	62·1 ± 2·0 (6)
40	23·1 ± 0·4 (4)	63·4 ± 3·2 (5)
100	25·2 ± 0·7 (6)	62·5 ± 1·3 (6)
200	24·4 ± 0·4 (5)	66·5 ± 2·5 (4)
400	24·3 ± 1·4 (4)	64·7 ± 0·8 (4)

* Values are the mean ± S.E. The number of rats is given in parentheses.
† CS was administered i.p. 20 min before $^2H_4$-choline injections.

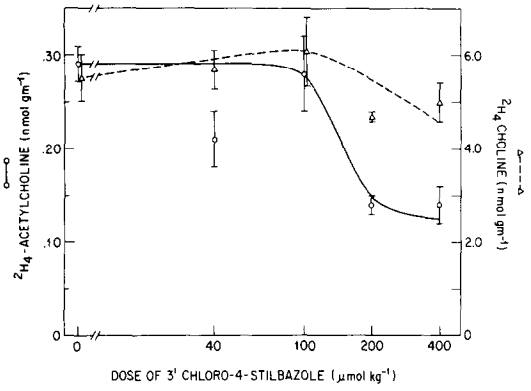

Fig. 3. Average rat brain concentrations of $^2H_4$-choline (right scale, △) and $^2H_4$-acetylcholine (left scale, ○) ± standard errors, 30 sec after the pulse intravenous injection of $^2H_4$-choline (20 μmoles kg^{-1}), as a function of the dose of CS given intraperitoneally 20 min before the choline.

ACh levels after repeated injections of atropine or atropine plus CS. Use of atropine to increase ACh turnover [6, 7] and an incubator to prevent the hypothermia previously noted by us [2] resulted in a 35 per cent decline in brain ACh in both atropine-treated and atropine +CS-treated animals ($t = 3{\cdot}836$ and 4·346, $P < 0{\cdot}01$ respectively; Table 3). Depletion of total ACh levels after i.p. atropine has been noted previously [8]. There was no significant difference between the two groups of atropine-treated animals ($t = 0{\cdot}655$, $P > 0{\cdot}05$).

ACh turnover in mice

In mice, brain levels of both ACh and Ch were moderately but significantly elevated at all doses of CS from 40 to 400 μmoles kg^{-1} (Table 4). These changes were independent of the sampling time within the range 20–50 min after CS, corresponding to 20 sec to 30 min after the pulse injection of $^2H_4$-Ch. The 20-sec curves of $^2H_4$-Ch or ACh vs CS (Fig. 4) resemble the 30-sec curves seen in rats (Fig. 3). Turnover of ACh was reduced after the higher dose levels

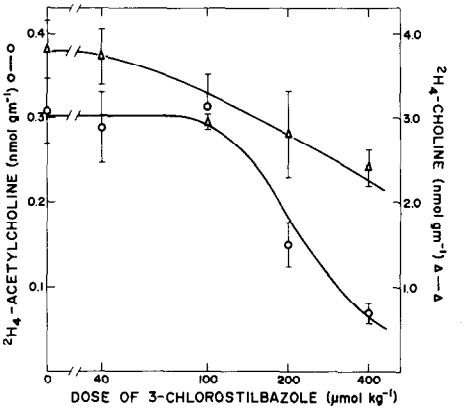

Fig. 4. Average mouse brain concentrations of $^2H_4$-choline (right scale, △) and $^2H_4$-acetylcholine (left scale, ○) ± standard errors, 20 sec after the pulse intravenous injection of $^2H_4$-choline (20 μmoles kg^{-1}), as a function of the dose of CS given intraperitoneally 20 min before the choline.

Table 2. Rat brain levels of acetylcholine and CS after repeated i.p. injections of CS

Animals	N	(nmoles ACh ± S. E./g brain)	(nmoles CS ± S. E./g brain)
Controls	6	17·8 ± 0·5	
CS	12	15·4 ± 0·6	342 ± 46

Table 3. Rat brain levels of acetylcholine and CS after repeated i.p. injections of atropine or atropine plus CS

Animals	N	(nmoles ACh ± S. E./g brain)	(nmoles CS ± S. E./g brain)
Controls	4	17·8 ± 0·9	
Atropine sulfate	6	11·9 ± 1·1	
Atropine sulfate and CS	6	11·6 ± 1·0	392 ± 65

Table 4. Brain levels of choline and acetylcholine after various doses of CS

Dose of CS† (μmoles kg^{-1})	Mean brain levels* Acetylcholine (nmoles g^{-1})	Choline (nmoles g^{-1})
0	15·1 ± 0·3 (111)	42·0 ± 1·0 (112)
40	17·9 ± 0·3‡ (31)	47·5 ± 1·4§ (32)
100	19·7 ± 0·3‡ (32)	47·4 ± 1·3§ (31)
200	16·8 ± 0·5§ (35)	48·9 ± 1·1‡ (35)
400	19·4 ± 0·6‡ (40)	53·7 ± 2·1‡ (39)

* Values are the mean ± S.E. The number of mice is given in parentheses.
† CS was given 20 min before $^2H_4$-choline injections.
‡ Significant difference from control ($P < 0.001$).
§ Significant difference from control ($P < 0.01$).

of CS, whether this was estimated from the uncorrected rate of formation of $^2H_4$-ACh after the pulse injection of $^2H_4$-Ch (Fig. 5) or whether this was corrected for changes in the specific activity of Ch in the brain (Fig. 6). The latter analysis revealed a significant increase in ACh turnover after lower doses of CS and a progressive decline at higher doses (Fig. 7).

DISCUSSION

The results presented here confirm previous reports [2, 9–11] that average brain ACh levels are not substantially reduced *in vivo* by prior administration of a potent ChA inhibitor.

The failure of CS to produce a significant decrement in brain acetylcholine levels was not due to the inability of this compound to cross the blood–brain barrier or to its pharmacokinetic properties. Analyses of plasma and brain showed that CS has a half-life in rats of about 3 hr, which is many times the estimated turnover time of ACh in brain. Moreover, the brain concentration was more than ten times the plasma concentration, indicating that CS not only crosses the blood–brain barrier but is concentrated

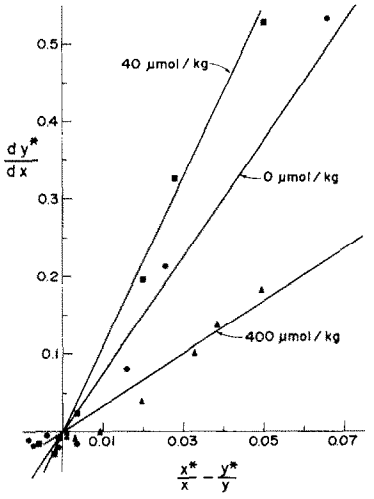

Fig. 6. Turnover estimates for mouse brain acetylcholine estimated according to Jenden *et al.* [5] after 0, 40 or 400 μmoles kg^{-1} CS given intraperitoneally 20 min earlier. Choline and acetylcholine concentrations are denoted by x and y, respectively; asterisks indicate $^2H_4$-labeled variants. Slopes of the lines provide estimates of mean acetylcholine turnover rates [5]. The ordinate was estimated by differentiation of an empirical curve relating y^* and time (t), and has the units nmoles g^{-1} min^{-1}. The abscissa is dimensionless.

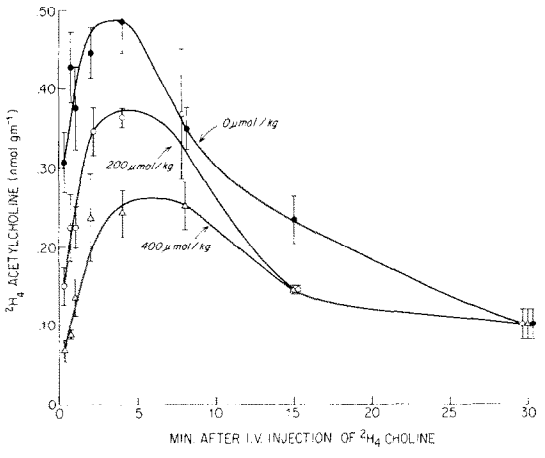

Fig. 5. Average mouse brain concentrations of $^2H_4$-acetylcholine ± standard errors at various times after the pulse intravenous injection of $^2H_4$-choline (20 μmoles kg^{-1}) after 0, 200 or 400 μmoles kg^{-1} CS given intraperitoneally 20 min prior to the choline.

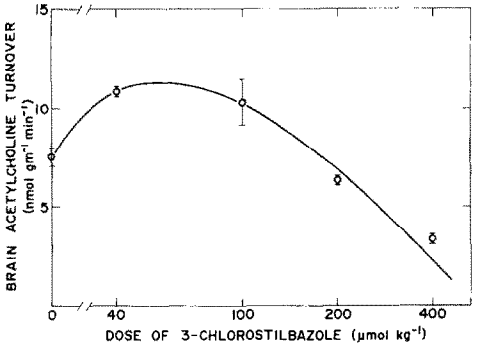

Fig. 7. Acetylcholine turnover in mouse brain as a function of CS dose administered intraperitoneally 20 min earlier. Turnover was estimated as the slopes of lines fitted through the origin, as in Fig. 6. Bars indicate standard deviations of the slopes.

in some component of the brain. Its identity was confirmed by comparison of the absorption spectra of brain extracts and authentic CS (Fig. 1).

CS remained in the brain far longer than the slowest estimate of ACh turnover (11 min) by Jenden *et al.* [5]. However, we examined the effects of repeated injections of CS, atropine and atropine plus CS. Even under these conditions the ACh levels were reduced 13–35 per cent. By contrast, hemicholinium-3 has been shown to produce an 80 per cent reduction in 2 hr [12].

The rates of $^2H_4$-ACh synthesis after i.v. $^2H_4$-Ch were compared in rats and mice 20 min after i.p. doses of CS ranging from 40 to 400 μmoles kg^{-1} (Figs. 3 and 4). Estimates of the turnover rate of brain ACh revealed the decrease which would be anticipated from ChA inhibition, although this only occurred after very large doses of CS. The increased rate of turnover after lower doses in mice is more difficult to explain. It is possible that this reflects an atropine-like action of CS [2]. Atropine is known to increase ACh release [6, 7], but the level of ACh would in that case be expected to fall. The weak anticholinesterase properties of CS may be significant in this context [1]. It is perhaps significant that the turnover rate appeared to increase only when the rate of ACh labeling in the brain was corrected for the decreased specific activity of Ch after CS. Since there is now no reliable way to estimate the specific activity of Ch in the pool from which ACh is synthesized, the average specific activity in the brain was used as an estimate of it. However, differential effects of a naphthylvinyl-pyridine analog have been reported on the high- and low-affinity Ch uptake systems [13], and it is possible that CS is also more effective against the nonspecific low-affinity Ch uptake. This would not impair labeling of the ACh synthesis pool of Ch to the degree that the decline in average specific activity of brain Ch suggests, and would account for the apparent increase in "corrected" but not absolute rate of synthesis of ACh which was observed.

Although some decrease in turnover rate of ACh was seen after large doses of CS, this was not as great as would be anticipated from the brain levels of CS which are obtained by extrapolation from the data on rats. Assuming comparable distribution in the two species, a brain concentration of 300 μM would be anticipated 30 min after a dose of 400 μmoles kg^{-1}. Since the I_{50} for CS and ChA is 7·8 μM, the enzyme should be almost completely inhibited if distribution of CS in the brain is uniform. We conclude that either this enzyme is not rate limiting under the conditions studied, or CS fails to gain access to the enzyme. In view of the lipophilic properties of CS, it seems unlikely that it fails to penetrate the cell membrane. Since ChA is a soluble enzyme [14], it would appear that CS can reach it, although this might be prevented by essentially irreversible binding to lipids. It seems more likely that, like several enzymes in the biosynthetic pathway for catecholamines, ChA is present in considerable excess and its partial inhibition is, therefore, without significant functional effect.

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