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INHIBITION OF ACETYLCHOLINE SYNTHESIS BY JUGLONE AND 4-(1-NAPHTHYLVINYL) PYRIDINE

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Abstract—Inhibition by juglone (5-hydroxy-1,4-naphthoquinone) of choline acetyltransferase [ChAt; acetyl-CoA: choline *O*-acetyltransferase (EC 2.3.1.6)] of rabbit brain was noncompetitive for choline and not reversible by dialysis. When acetyl-CoA and juglone were mixed together before the addition of ChAt, inhibition was competitive with respect to acetyl-CoA; when juglone was preincubated with the enzyme, inhibition was mixed competitive and noncompetitive. The effects *in vivo* of juglone and 4-(1-naphthylvinyl) pyridine (NVP) were determined in brains of mice sacrificed by microwave radiation. Changes in the rate of synthesis of acetylcholine were assessed by measuring the amount of choline-methyl[^{3}H] converted to acetylcholine in the brains of mice 30 sec after the intravenous administration of the isotope. Administration of two doses of juglone (2 mg/kg, 1 hr apart) increased the concentrations of both endogenous and radioactive choline in brain, but had no effect on either the rate of synthesis or the concentration of acetylcholine. These results suggest that juglone, when administered to animals, does not inhibit brain ChAt. On the other hand, administration of two doses of 4-(1-naphthylvinyl) pyridine (100 mg/kg, i.p., 1 hr apart) did inhibit the synthesis of acetylcholine, but had no effect on the concentration of either choline or acetylcholine in brain. This latter finding indicates that inhibition of ChAt can occur *in vivo* without causing a reduction in the concentration of acetylcholine.

None of the known inhibitors *in vitro* of choline acetyltransferase [ChAt; acetyl-CoA: choline *O*-acetyltransferase (EC 2.3.1.6.)] causes a decrease in the concentration of acetylcholine in the brain when administered to animals [1–5]. The inability of these inhibitors to lower the concentration of acetylcholine may be due to their failure to inhibit ChAt *in vivo*, or to their causing a simultaneous decrease in the rate of turnover of acetylcholine. The present study shows that juglone, an extract of walnut hulls with depressant activity in animals [6], inhibits ChAt *in vitro*. Treatment of mice with juglone had no effect on either the synthesis of acetylcholine or its concentration in brain, whereas administration of 4-(1-naphthylvinyl) pyridine (NVP), another inhibitor of ChAt, did cause a decrease in the rate of synthesis of brain acetylcholine.

EXPERIMENTAL

Chemical assays. Choline acetyltransferase activity was assessed by measuring the rate of conversion of choline to acetyl[^{14}C]choline in the presence of acetyl[^{14}C]CoA (2–72 μM, New England Nuclear), choline (0·1 to 4 μM), potassium phosphate buffer (10 mM, pH 7·4), KCl (200 mM), EDTA (1·0 mM), bovine serum albumin (0·1%, w/v), Triton X-100 (0·1% v/v) and the aqueous extract of 25 μg (dry weight) of rabbit brain acetone powder (Pel-Freez Biologicals). The mixture was incubated for 10 min at 37° in a volume of 25 μl. The reaction was stopped by the addition of 5 μl of 1 N formic acid, with mixing and chilling to 0–2°. Acetyl[^{14}C]choline was isolated by high-voltage paper electrophoresis, as previously described [7, 8], and measured by liquid scintillation spectrometry. Juglone was dissolved in dimethylsulfoxide (DMSO) so that the final concentration of DMSO during incubation was 2% (v/v).

Choline and acetylcholine were isolated from brain by high-voltage paper electrophoresis and measured by an enzymatic radioisotopic procedure [7, 8]. Concentrations of radioactive and endogenous choline and of acetylcholine were determined simultaneously in mouse brain, as previously described [9].

Dialysis. Juglone (100 μM) was mixed with the aqueous extract of a rabbit brain acetone powder (5 mg powder/ml of 200 mM KCl) and incubated for 5 min at 0–4°. The mixture was then dialyzed at 0–4° for 18 hr against 1000 vol. of 10 mM potassium phosphate buffer (pH 7·4) containing 200 mM KCl.

Treatment of mice. Male white mice (20–25 g) were obtained from Zartman Farms, Douglasville, Pa. Juglone (Aldrich) or 4-(1-naphthylvinyl) pyridine HCl (CalBiochem) was suspended in 0·9% NaCl containing a few drops of Tween 80. Two doses of juglone (2 mg/kg, i.p.; 1 ml/kg) or of NVP (100 mg/kg, i.p.; 1·25 ml/kg) were administered 1 hr apart. Control mice were treated with the vehicles used to dissolve the compounds. Choline-methyl[^{3}H] (New England Nuclear) was administered (2·5 mCi/kg, i.v.) 1 hr after the second dose of each drug, and the mice were sacrificed 30 sec thereafter. Mice were killed by microwave irradiation from a microwave radiator fitted with a wave guide (C. Wang, Medical Engineering Consultants, Lexington, Mass.). The total power focused down the wave guide and onto the head was 1300 W at a frequency of 2450 MHz. Mice were exposed to the radiation for 3·0 to 3·6 sec. Some of the mice treated with juglone or NVP were decapitated, and brains were removed and frozen by dropping the tissue into a beaker of acetone on solid CO_2.

RESULTS

Figure 1 shows the effect of choline on the inhibition of ChAt by juglone (25 μM). Juglone reduced the

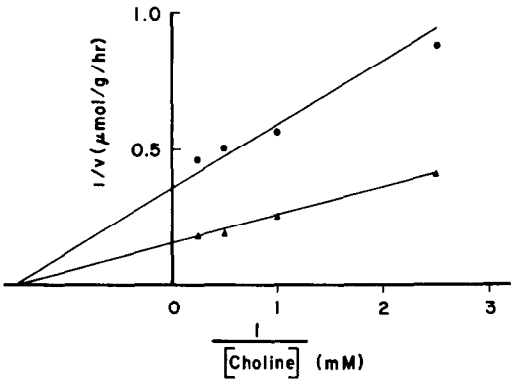

Fig. 1. Double-reciprocal plot of the velocity of choline acetyltransferase as a function of the concentration of choline (0·1 to 4·0 mM), measured in the absence (▲) or presence (●) of juglone (25 μM). Activity was measured in the acetone powder of a rabbit brain immediately after mixing juglone with the enzyme. The acetyl-coenzyme A concentration was 73 μM.

maximal velocity of ChAt, but had no effect on its affinity for choline. In this experiment, the enzyme was added to the incubation tubes immediately after the addition of juglone and the substrates. When a similar experiment was performed to evaluate the effect of the concentration of acetyl-CoA on inhibition by juglone, the inhibitor increased the K_m for acetyl-CoA without affecting the V_{max} (Fig. 2). However, when the enzyme and inhibitor were incubated together for 16 hr (2°) and the velocity was measured as a function of concentration of acetyl-CoA, juglone both reduced the V_{max} and increased the K_m for acetyl-CoA (Fig. 3).

Inhibition of ChAt by juglone was not reversible by dialysis. When ChAt inhibited by 100 μM juglone was dialyzed to remove the inhibitor, enzyme activity was reduced 82 per cent, as compared to 75 per cent inhibition of the undialyzed enzyme. Dialysis had no significant effect on control enzyme activity.

The effects of administration of juglone and NVP on the concentration of choline and acetylcholine in brain are shown in Table 1. Treatment of mice with either inhibitor of ChAt had no significant effect on the concentration of acetylcholine when the animals were sacrificed either by decapitation or by microwave irradiation. The administration of juglone did, however, induce a significant increase in the concentration of free choline in brains of mice, but this was evident only when the mice were killed by irradiation.

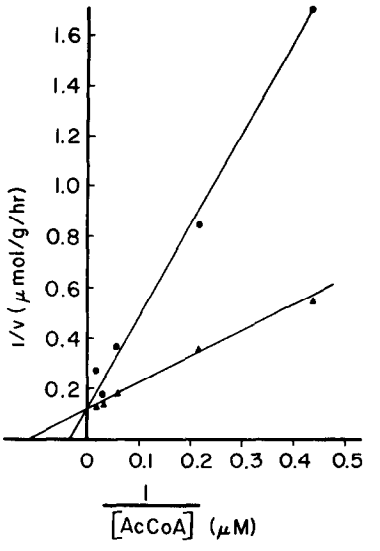

Fig. 2. Double-reciprocal plot of the velocity of choline acetyltransferase as a function of the concentration of acetyl-coenzyme A (2·28 to 73 μM), measured in the absence (▲) or presence (●) of juglone (25 μM). Choline concentration was 4 mM. Activity was measured as described in Fig. 1.

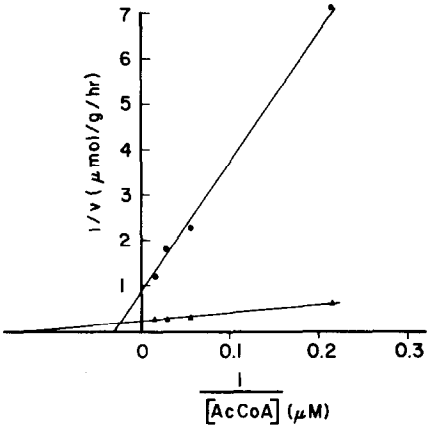

Fig. 3. Double-reciprocal plot of the velocity of choline acetyltransferase as a function of the concentration of acetyl-coenzyme A. The experiment was similar to that described in Fig. 2, except that juglone was preincubated with the enzyme at 2° for 16 hr prior to addition of substrates. Control (▲); juglone (●)

Table 1. Effect of 4-(1-naphthylvinyl) pyridine or juglone on choline and acetylcholine in mouse brain

	Choline		Acetylcholine	
Treatment*	Decapitation (nmoles/g ± S. E.	Irradiation (nmoles/g ± S. E.)	Decapitation (nmoles/g ± S. E.)	Irradiation (nmoles/g ± S. E.)
Vehicle	40 ± 3	28 ± 3	12·1 ± 0·5	29 ± 3
Naphthylvinyl pyridine	38 ± 1	39 ± 5	10·6 ± 0·6	24 ± 3
Juglone	39 ± 2	46 ± 5†	13·0 ± 1·0	33 ± 1

* Two doses of 4-(1-naphthylvinyl) pyridine HCl (NVP) (100 mg/kg) or juglone (2 mg/kg) were administered intraperitoneally to mice, 1 hr apart. One hr after the last dose of NVP or juglone, the mice were sacrificed, either by decapitation or by microwave irradiation, and their brains frozen on solid CO_2. Each group contained five or six mice.

† $P < 0·05$, Student's t-test.

Table 2. Effect of 4-(1-naphthylvinyl) pyridine or juglone on acetylcholine synthesis in mouse brain

Treatment*	Specific activity Choline (dis./min/ nmole ± S.E.)	Acetylcholine (dis./min/ nmole ± S.E.)
Vehicle	19,150 ± 2,230	5,780 ± 660
Naphthylvinyl pyridine	16,960 ± 2,280	2,810 ± 310†
Juglone	23,480 ± 2,450	5,225 ± 403

* Mice were treated as described in Table 1. Choline-methyl[^{3}H] (2·5 mCi/kg) was administered intravenously 1 hr after the last dose of juglone or NVP. Mice (N = 5 or 6) were sacrificed by microwave irradiation 30 sec after administration of the isotope.

† $P < 0{\cdot}001$, Student's *t*-test.

Treatment with NVP had no significant effect on the concentration of free choline in brains of mice killed by either method.

When mice were killed by microwave irradiation, the concentration of choline was lower, and that of acetylcholine higher, than in the brains of mice that had been decapitated (Table 1). The concentrations of choline and acetylcholine in brains of mice are nearly identical to those of rat brain, as reported by Stavinoha and Weintraub [10], who also used microwave irradiation to cause death. In our laboratory, the activity of brain ChAt was completely inactivated by microwave irradiation (unpublished results).

As shown in Table 2, treatment of mice with NVP significantly reduced the incorporation of intravenously administered choline-methyl-[^{3}H] into acetylcholine in brains, such that the specific activity of acetylcholine in brains of mice treated with NVP was less than half that in the control animals. On the other hand, juglone had no effect *in vivo* on the rate of synthesis of acetylcholine (Table 2). Administration of juglone did increase the amount of radiolabeled choline present in the brain, but because the concentration of endogenous choline was also elevated (see Table 1), there was no significant change in the specific activity of the precursor (Table 2).

DISCUSSION

The results of these studies demonstrate that juglone inhibited *in vitro* the activity of ChAt. The inhibition was noncompetitive for choline, and of the mixed competitive and noncompetitive type for acetyl-CoA when the enzyme had been preincubated with juglone in the absence of substrates. However, when ChAt was added to incubation tubes containing both acetyl-CoA and juglone, and enzyme activity was measured immediately, the plot of $1/v$ vs $1/[s]$ revealed that the inhibition was competitive. This result suggests that acetyl-CoA protects the enzyme from juglone by slowing the rate of inhibition. Therefore, juglone might inhibit ChAt by combining with the site on the enzyme that normally binds acetyl-CoA. Juglone could inhibit by binding to a sulfhydryl group on the enzyme, an interpretation consistent with the known ability of quinones to react covalently with thiols [11], with the finding that inhibition of ChAt by juglone is irreversible by dialysis, and with the possibility that the active site of ChAt contains a sulfhydryl group that binds acetyl-CoA [12].

The failure of juglone to lower the concentration of acetylcholine was probably caused by a lack of inhibition of ChAt when the inhibitor was administered to mice. This conclusion is consistent with other studies in our laboratory (unpublished) showing that the activity of ChAt measured in the brains of mice treated with juglone was not different from that of the controls. Quinones are known to bind to plasma proteins [13], and this binding might have prevented juglone from entering cholinergic neurons.

The increase in concentration of both endogenous and radiolabeled choline in the brains of mice treated with juglone and subsequently killed by irradiation might have been caused by an effect of the compound on the metabolism of choline outside the brain. The juglone-induced increase in endogenous choline was undetectable in mice killed by decapitation possibly because of the large postmortem increase in concentration of free choline.

The inability of NVP to lower the concentration of acetylcholine in brain is probably not caused by the failure of the compound to inhibit ChAt after its administration to animals. Treatment of mice with NVP did induce a decrease *in vivo* in the rate of acetylcholine synthesis (Table 2). Similar inhibition *in vivo* of acetylcholine synthesis has been reported by Saelens *et al.* [14]. Furthermore, Carson *et al.* [2] have shown by direct assay of chlorostilbazole, an analog of NVP, that the compound does enter the brain in amounts sufficient to inhibit ChAt. In addition, Krell and Goldberg [4] have found that treatment of mice with NVP accelerates the depletion of brain acetylcholine that occurs during stress, and reduces the rate of accumulation of brain acetylcholine induced by prior administration of pentobarbital. Furthermore, the activity of ChAt measured *in vitro* in brains taken from animals treated with NVP is inhibited more than 60 per cent as compared with controls [4, 15]. Thus, it appears that NVP inhibits *in vivo* the synthesis of acetylcholine.

NVP might fail to reduce the concentration of acetylcholine in brain because the inhibitor causes a simultaneous decrease in the rate of turnover of the neurotransmitter. Direct inhibition of acetylcholine esterase by NVP probably does not occur, because the compound is only a weak inhibitor *in vitro* of this catabolic enzyme [16]. The possibility exists, therefore, that NVP prevents the release of acetylcholine and simultaneously causes a decrease in its rate of synthesis such that steady state levels of the neurotransmitter are maintained. Such an effect would ultimately lead to a central anticholinergic action and could account for the similarities between acetylcholine-receptor blockers and NVP in reducing the turnover of dopamine in the corpus striatum [17] and in producing certain behavioral effects [3]. This postulated ability of NVP to reduce the turnover of acetylcholine could be a property of the compound unrelated to its ability to inhibit ChAt. On the other hand, if synthesis and utilization of the acetylcholine are interdependent processes, the rate of turnover of the neurotransmitter might be reduced because its synthesis in inhibited.

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