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CHOLINE ACETYLTRANSFERASE

ENZYME MECHANISM AND MODE OF INHIBITION BY A STYRYLPYRIDINE ANALOGUE

H. L. WHITE AND C. J. CAVALLITO
School of Pharmacy, University of North Carolina, Chapel Hill, N.C. (U.S.A.)

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SUMMARY

1. Choline acetyltransferase of the calf caudate nucleus was studied, with particular emphasis on its mode of inhibition by styrylpyridine analogues.

2. Product-inhibition studies suggest a sequential, Theorell–Chance type of mechanism for this enzyme. CoA was competitive with the substrate acetyl–CoA, and acetylcholine was competitive with choline.

3. Inhibition by styrylpyridine analogues was non-competitive with both acetyl–CoA and choline and was initially reversible, but partial irreversibility was observed when the enzyme and inhibitor were preincubated at 37° before substrate addition.

4. Acetylcholinesterase was also inhibited to various degrees by most of the styrylpyridine analogues, and with this enzyme, *N*-methyl-4-(1-naphthylvinyl)pyridinium iodide (NVP^+) was found to block the enzyme deacylation step (E–acetyl + $H_2O \rightleftarrows E$ + acetic acid), which is thought by other workers to involve an imidazole moiety on cholinesterase.

5. With choline acetyltransferase in the presence of [^{14}C]acetyl–CoA, an apparent [^{14}C]acetyl–CoA–enzyme intermediate was detected by gel filtration. The relative amount of this intermediate was diminished when choline was present. NVP^+ did not prevent its formation, but did prevent its disappearance in the presence of choline. This effect with the acetyltransferase may be analogous to the blocking of deacylation observed with acetylcholinesterase.

6. The inhibition of choline acetyltransferase by NVP^+ was not reversed by dithiothreitol, although this sulfhydryl compound completely reversed inhibition by Hg^{2+} and by the specific sulfhydryl reagent, allyl 2-propene-1-thiolsulfinate (allicin). Inhibition by Cu^{2+}, which may be expected to interact with imidazole groups as well as others, tended to protect the enzyme from NVP^+. It is proposed that NVP^+ may interfere with the functioning of a catalytic imidazole moiety during enzyme deacylation.

Abbreviation: NVP^+, *N*-methyl-4-(1-naphthylvinyl)pyridinium iodide.

INTRODUCTION

Choline acetyltransferase (acetyl–CoA:choline *O*-acetyltransferase, EC 2.3.1.6) catalyzes the synthesis of acetylcholine from acetyl–CoA and choline. The most potent known inhibitors of this enzyme are a group of *trans*-styrylpyridine analogues[1–4]. A study of the mechanism by which these compounds inhibit choline acetyltransferase was undertaken, using an extract of enzyme obtained from the calf caudate nucleus and the inhibitor shown in Fig. 3. Since very little was known about the mechanism of the enzyme, certain kinetic properties of the uninhibited enzyme also were studied. Most of the styrylpyridine analogues appear to inhibit acetylcholinesterase to a lesser degree (acetylcholine hydrolase, EC 3.1.1.7), and therefore it was of interest to investigate this latter system. A possible mode of inhibitory action which implicates a catalytic imidazole in both enzyme systems is proposed.

EXPERIMENTAL PROCEDURES

Choline acetyltransferase extract from the calf caudate nucleus was kindly provided by Dr. F. F. Foldes of Montefiore Hospital and Medical Center, Bronx, N.Y. A 100 000 $\times g$ supernatant was fractionated with $(NH_4)_2SO_4$. Our preparation was a 30–40% fractionation-cut. Different batches had specific activities in our laboratory of 2–4 nmoles/min per mg protein at 37°. Acetylcholinesterase of *Electrophorous electricus* (purified by chromatography and gel filtration) with a specific activity of 1100 I.U./mg (μmoles/min per mg protein) and bovine pancreas α-chymotrypsin with activity of 45 I.U./mg were purchased from Worthington Biochemical Corp.

[^{14}C]Acetyl–CoA (60 C/mole, radiochemical purity 95%) was purchased from New England Nuclear Corp.; unlabeled acetyl–CoA (93%), bovine albumin (electrophoretically pure), eserine sulfate, and *p*-nitrophenyl acetate from Mann Research Laboratories; acetylcholine bromide from Sigma Chemical Co.; choline chloride from Merck and Co.; dithiothreitol from Calbiochem. Phenyl acetate from Will Corp. was repeatedly extracted with water to remove phenol and then distilled under vacuum immediately before use.

N-Methyl-4-(1-naphthylvinyl)pyridinium iodide (NVP^+) was synthesized as described[1]. Because NVP^+ and its analogues are very easily photoisomerized from *trans*- to *cis*-forms[2,4], all work with this compound was performed with illumination by a 75-W General Electric pink light bulb.

Allyl 2-propene-1-thiolsulfinate (allicin) was prepared from an extract of garlic[5]. 4-Aminomethylstilbene was synthesized in our laboratory[6]. Other reagents were of commercially available reagent grade. Glass-distilled water was used for all solutions.

Assays of enzyme activity

The enzymatic transfer of the acetyl moiety from [^{14}C]acetyl–CoA to choline was followed using an assay described elsewhere[4]. Assay components and concentrations were: [^{14}C]acetyl-CoA, 0.01–0.1 mM; choline, 0.1–5 mM; bovine plasma albumin, 0.05%; NaCl, 0.3 M; potassium phosphate, 0.07 M (pH 6.7). All assays were done in duplicate or triplicate. Eserine at 0.2 mM as an acetylcholinesterase inhibitor was found to be unnecessary with our extract.

Activity of eel acetylcholinesterase with phenyl acetate and *p*-nitrophenyl acetate as substrates was assayed spectrophotometrically, using a Cary Model-15

spectrophotometer with synchronous drive, as described in Table III. The procedure was a modification of that of PURDIE[7]. The enzyme stock solution was prepared at 50 I.U./ml in glass-distilled water containing 20 mM $MgCl_2$ and 0.05% bovine albumin and stored at 5°. Substrates were first dissolved in absolute ethanol and added in μl amounts with a Hamilton microsyringe to sample and reference cuvettes which contained 0.1 M sodium phosphate buffer (pH 7.8), or an inhibitor dissolved in the buffer. Enzyme was present only in the sample cuvette. With phenyl acetate, enzyme concentration was 0.080 I.U./ml, and with the poorer substrate, *p*-nitrophenyl acetate, 0.40 I.U./ml.

Scintillation counting

Samples were counted in a Nuclear Chicago Mark I or a Packard Tricarb scintillation spectrometer. On Whatman No. 1 chromatography paper strips, ^{14}C gave a counting efficiency of 54% in a scintillation fluid containing 5 g/l of 2,5-diphenyloxazole. Aqueous eluants from Sephadex columns were counted at 72% efficiency in a toluene–Triton X-100 mixture (2:1, by vol.) containing 4 g/l of 2,5-diphenyloxazole[8].

Sephadex techniques

A Pharmacia K9/15 analytical size column was packed with Sephadex G-100 (inner bed volume = 4.9 ml) and equilibrated at 5° with 0.07 M potassium phosphate buffer (pH 6.7). Samples of 0.20 ml were layered over the bed, and elution was carried out with the same phosphate buffer. Further details are in Table II.

Spectrophotometry

Cary recording spectrophotometers, Models 14 and 15, were used for all absorption spectroscopy. Spectrophotometric properties of NVP^+ and related compounds have been described in an earlier publication[4].

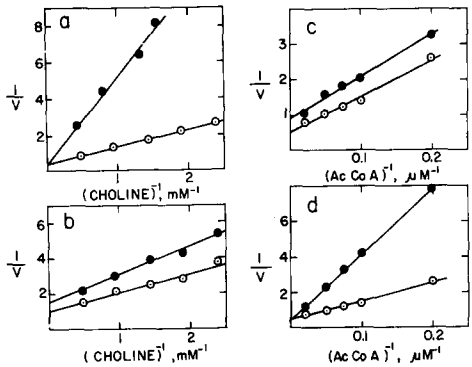
Dialysis

Visking 8/100 dialysis membrane (Union Carbide) was pretreated by boiling for 5 min in 0.1% disodium EDTA and then stirred in glass-distilled water at 5° for 24 h with three solvent changes, before being soaked in the dialysis buffer. This procedure was necessary to remove from the membranes substances such as Cu^{2+}, which are inhibitory to the acetyltransferase.

RESULTS

Enzyme mechanism

Choline acetyltransferase catalyzes the reaction of two substrates, acetyl–CoA and choline, to form two products, acetylcholine and CoA, and therefore is bireactant in both directions. Initial-velocity patterns obtained by varying the concentration of each substrate in the presence of fixed concentrations of the other clearly distinguish between sequential and ping-pong mechanisms. Plots of the reciprocal of initial velocity *versus* reciprocal of the varying substrate concentration determined at several different concentrations of the other substrate will give a series of parallel lines only for the latter case[9]. With choline acetyltransferase of calf caudate nucleus, such reciprocal plots always intersected to the left of the ordinate, indicating a



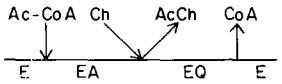


Fig. 1. Product inhibition of choline acetyltransferase. ○, uninhibited; ●, + product inhibitor. a. Inhibition by 33 mM acetylcholine with choline as variable substrate. Acetyl–CoA, 50 μM. Acetylcholine was contaminated with 0.7% choline which was determined by assays containing no added choline. Initial velocities and choline concentrations were therefore corrected. (b). Inhibition by 10 μM CoA with choline as variable substrate. Acetyl–CoA, 10 μM. c. Inhibition by 100 mM acetylcholine with acetyl–CoA as variable substrate. Choline, 5.0 mM. d. Inhibition by 20 μM CoA with acetyl–CoA as variable substrate. Choline, 5.0 mM. The 'least squares' method was used in drawing lines.

Fig. 2. Theorell–Chance mechanism using the notation of CLELAND[9], as it may apply to choline acetyltransferase on the basis of product inhibition studies. E = free enzyme; EA and EQ are transitory enzyme complexes; Ch = choline; Ac = acetyl.

sequential mechanism, similar to that reported for rat brain enzyme by POTTER *et al.*[10] Apparent K_m values, extrapolated to saturating values of both substrates, were 16 μM for acetyl–CoA and 0.8 mM for choline. Hill plots[11,12] indicated that only 1 molecule of each substrate is required per enzyme active center.

Further information about the enzyme mechanism was obtained from product-inhibition studies, examples of which are shown in Fig. 1. Since each product is competitive with only one substrate, and since reciprocal plots give straight lines, a random mechanism is improbable, and the addition of substrates to the enzyme occurs in a precise order. The product-inhibition mechanisms (Table I) are consistent with only one of the bi-bi ordered mechanisms discussed by CLELAND[9]—the Theorell–Chance mechanism. These experiments show that acetyl–CoA and CoA are a competitive

TABLE I

PRODUCT INHIBITION OF CHOLINE ACETYLTRANSFERASE

Acetyl–CoA was varied from 0.01 to 0.1 mM and choline from 0.1 to 3 mM. When acetyl–CoA was varied, choline concentration was held constant at 5.0 mM. When choline was varied, acetyl–CoA was constant at either 0.010 or 0.10 mM. All mixing was done at 5°, and incubation was at 37° for 10 min (ref. 4).

Product	*Variable substrate*	*Inhibition pattern*
Acetylcholine	Acetyl–CoA	Non-competitive
Acetylcholine	Choline	Competitive
CoA	Acetyl–CoA	Competitive
CoA	Choline	Non-competitive

substrate–product pair and that choline and acetylcholine are another such pair. They do not determine which substrate binds first.

There are various reasons for believing that acetyl–CoA binds to the enzyme as the first substrate, as shown schematically in Fig. 2: (a) Acetyl–CoA is a coenzyme. (b) Acetyl–CoA probably binds more strongly than choline as indicated by a 50-fold difference in K_m values for the two substrates. (c) Acetyl–CoA tends to stabilize the enzyme against loss of activity on standing at 5°. (d) An apparent [^{14}C]acetyl–CoA–enzyme intermediate was detected in the absence of choline (to be discussed in a later section). The proposed mechanism should be considered tentative until confirmed with more highly purified enzyme.

Product-inhibition studies indicated that inhibition by CoA was much stronger than that by acetylcholine. The apparent K_i for acetylcholine competition with choline was about 8 mM, while the apparent K_i for CoA inhibition, with varying acetyl–CoA and saturating choline concentrations, was 7.5 ± 1.5 μM. The apparent dissociation constant for acetyl–CoA binding to the enzyme was 7.4 ± 0.7 μM. This last value was determined from the point of intersection of reciprocal plots, with varying acetyl–CoA at several fixed choline concentrations, and is the K_{ia} value discussed by CLELAND[9]. The similarity of these two experimental values and the competitive product inhibition kinetics suggest that, at least under our assay conditions, acetyl–CoA and CoA exhibit similar affinities for the same enzyme form. The acetyl moiety may therefore not participate in initial binding of acetyl–CoA to the enzyme.

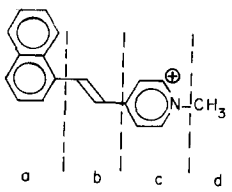
Nature of inhibition of choline acetyltransferase by NVP$^+$

NVP$^+$ (Fig. 3) inhibited the formation of acetylcholine by caudate nucleus choline acetyltransferase with an apparent K_i under our conditions of 0.55 μM, determined by extrapolating experimental K_i values to saturating concentrations of both substrates. The inhibition mechanism, ascertained from reciprocal plots similar to those in Fig. 1, was non-competitive with both acetyl–CoA and choline. No evidence of an allosteric effect could be detected in initial-velocity studies. The coplanar *trans*-form of NVP$^+$ shown in Fig. 3 is the inhibitor, and if partial photoisomerization to the *cis*-form is allowed to occur, inhibitory potency decreases proportionately[2,4]. Structural parameters of styrylpyridine-type compounds which favor inhibitory activity against choline acetyltransferase have been discussed[1,3].

Fig. 4 is a Hill plot showing inhibition by *N*-hydroxyethyl-4-(1-naphthylvinyl)-pyridinium iodide, a more water-soluble analogue of NVP$^+$. The shape of the curve in this plot was typical for inhibition by all the styrylpyridine analogues. The slope is 1.04 at low inhibitor concentrations, indicating that only 1 inhibitor molecule is bound per active center[13]. The decrease in slope at higher inhibitor concentrations suggests that the inhibited enzyme may still retain partial activity. Other explanations are possible. For example, higher concentrations of inhibitor may cause configurational changes in the enzyme. In addition, stacking of inhibitor molecules may occur on protein surfaces at higher concentrations.

Reversibility

That the inhibition is initially reversible was demonstrated by dilution experiments, in which inhibition decreased as inhibitor was diluted, and by full recovery



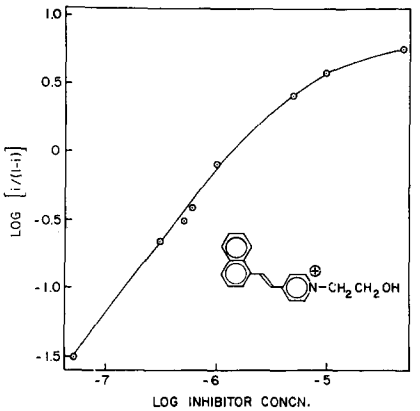


Fig. 3. Structure of *trans*-*N*-Methyl-4-(1-naphthylvinyl)pyridinium iodide (NVP+). Other styryl-pyridine-type inhibitors of choline acetyltransferase are analogues of this compound in which: a may be phenyl, naphthyl, phenanthryl, or certain substituted derivatives of these; b, a double or triple bond; c, a pyridine or quinoline moiety; d, methyl, hydroxyethyl, or various other moieties[1,3].

Fig. 4. Hill plot[11,12] for inhibition of choline acetyltransferase. $i = 1 - v_i/v_0$, where v_0 and v_i are initial velocities in absence and presence of inhibitor respectively. Molar I_{50} concentration of inhibitor is determined at log $[i/(1 - i)] = 0$.

of enzyme activity after elution of an enzyme–inhibitor solution through Sephadex G-100 at 5°. A concentration of inhibitor which normally caused 75% inhibition of the enzyme was preincubated with the extract for 30 min and then passed through Sephadex G-100 with an eluting buffer consisting of 0.07 M potassium phosphate buffer (pH 6.7) containing 0.1 mM dithiothreitol. When the 30-min preincubation step was carried out at 5°, the recovered enzyme was fully active, compared with a control experiment in which the enzyme had not been treated with inhibitor. However, if the 30-min preincubation step was performed at 37°, the recovered enzyme exhibited only 70% of its original activity, compared with an uninhibited portion of extract which was submitted to the same procedure. That is, an irreversible effect had been introduced which resulted in about 30% inhibition.

Another type of experiment was designed to further study the effect of preincubation at 37°. Portions of enzyme extract were added to either 0.07 M potassium phosphate buffer (pH 6.7) or to the same buffer containing NVP+ sufficient to give 50% inhibition. These were incubated at 37°, and at intervals aliquots were removed for assay. As expected, the activity of the uninhibited extract decreased slowly to about 81% of its original value after 30 min preincubation. The activity of the partially-inhibited enzyme was stable for at least 10 min, but suffered a rapid loss in activity between 10 and 30 min. Thereafter the inhibited enzyme lost activity at about the same rate as the uninhibited control. The inhibition was increased from 50% at zero time to 74% after 30 min preincubation at 37°. In each case the calculated inhibition was based on the activity of uninhibited enzyme preincubated for the same length of time. The difference between 50 and 74% probably represents the irreversible component of the inhibition which was observed after Sephadex elution of a 37° preincubated enzyme–inhibitor mixture.

Exhaustive dialyses of enzyme solutions in the absence and presence of concentrations of NVP^+ which would produce 50–80% inhibition were carried out at 5° for 20 h. Dialysis was performed against 0.07 M potassium phosphate buffer (pH 6.7), against the same buffer containing 0.1 mM dithiothreitol or 0.1 mM EDTA, or with both dithiothreitol and EDTA. The last solvent most effectively preserved the activity of uninhibited enzyme. In no instance was significant reversal of inhibition obtained, although a 17 000-fold dilution of inhibitor was theoretically achieved. Separate experiments ascertained that the dialysis membranes were permeable to NVP^+. During dialysis, changes in enzyme configuration may have occurred which resulted in potentiation of irreversible inhibition.

The normal incubation conditions used in most activity assays were 10 min at 37°. Since irreversibility was not detectable during the first 10 min of preincubation of enzyme with inhibitor at 37°, it may be assumed that the usual kinetic experiments were not significantly influenced by the irreversible component of inhibition. However, this irreversibility may be an important consideration in the use of NVP^+ and its analogues in intact animals.

A possible acetyl–CoA–enzyme intermediate

According to the proposed mechanism shown in Fig. 2, in the presence of acetyl–CoA and no choline, one might expect a build-up in the concentration of one or more intermediates, which are symbolized by *EA* in the diagram. If these are sufficiently stable, it might be possible to detect them by gel filtration. When excess choline is added, the concentration of *EA* should diminish as the product, acetylcholine, is formed. The presence of an inhibitor in the system might either prevent the formation of *EA* or hinder its breakdown when choline is added.

Table II shows the results of such an experiment. With no inhibitor, there was more extract-bound [^{14}C]acetyl (either as acetyl–CoA–enzyme or acetyl–enzyme) than when choline was added. However, the extract-bound acetyl was not reduced to zero by choline. This is not surprising, since acetyl–CoA binding proteins other than choline acetyltransferase probably were present in the extract. Exhaustive dialysis of similar

TABLE II

EFFECT OF NVP^+ ON AN APPARENT ^{14}C-LABELED ENZYME INTERMEDIATE

Aliquots (1 mg extract protein in 0.20 ml) of the caudate nucleus extract contained acetyl–CoA, 15.7 μM (60.7 C/mole); potassium phosphate buffer, 35 mM; dithiothreitol, 0.1 mM; NaCl, 0.3 M; bovine plasma albumin, 0.05%; pH = 6.7. Where present, choline was 5 mM and NVP^+ was 23 μM. After incubation at 30° for 30 min, these aliquots were passed through Sephadex G-100 at 5°. Eluting solvent was 0.07 M potassium phosphate buffer (pH 6.7). For each gradient 20 fractions of 10 drops were collected manually directly into scintillation vials. The peak of activity for [^{14}C]acetyl–CoA or [^{14}C]acetylcholine appeared in Fractions 11–13. A much smaller peak occurred in Fraction 3, which is the first fraction after the void volume and contains the enzyme. Counts/min below are the sums of fractions 1–5.

	Amount of ^{14}C in enzyme-containing fractions (counts/min)		
	−choline	*+ choline*	*Δ*
Control	1284	587	697
NVP^+	1309	1214	95

preparations at 5° showed that 250–300 counts/min were irreversibly bound to this amount of extract whether or not choline was present.

The last column in Table II gives the acetyl–extract activity which was removed by addition of choline, and therefore reflects the activity of choline acetyltransferase. When NVP^+ was present, the formation of acetyl–extract was not inhibited, but the breakdown of an enzyme intermediate on choline addition appeared to be inhibited.

Other similar experiments demonstrated that the amount of [^{14}C]acetyl–CoA bound by the extract was proportional to the concentration of extract. The bound acetyl which was removable by choline appeared to be relatively unstable to prolonged incubation at 30°, but the inhibitor in all cases tended to stabilize the *EA* complex.

From the enzyme-bound radioactivity, a minimum turnover number of 400 moles/min was calculated, assuming an enzyme molecular weight of 10^5 (ref. 43). An estimated turnover number of 660–1250 moles/min has been reported for choline acetyltransferase[14].

Inhibition of acetylcholinesterase

Styrylpyridine analogues are capable of inhibiting acetylcholinesterase in addition to choline acetyltransferase[1,3,4], although the two enzyme systems are not similarly influenced by structural changes among the inhibitors. Acetylcholinesterase is believed to form a relatively stable acetyl–enzyme intermediate, analogous to that of chymotrypsin, in which the acetyl is covalently bound to an enzymic serine moiety, which is catalytically hydrolyzed to give the free enzyme and acetic acid[15–18].

(a) E + acetylcholine $\rightleftarrows$ E–acetyl + choline

(b) E–acetyl + H_2O $\rightleftarrows$ E + acetic acid

This latter enzyme deacylation step is rate-determining when either acetylcholine or phenyl acetate is the substrate[7]. It is apparent that any compound which inhibits primarily the enzyme deacylation step (b) will exhibit a similar K_i value with any substrate which can produce an acetyl–enzyme intermediate. Table III shows that the hydrolysis of three different substrates by acetylcholinesterase is inhibited by

TABLE III

INHIBITION OF ACETYLCHOLINESTERASE BY NVP^+

Acetylcholinesterase from *E. electricus* was used in all assays. With acetylcholine as substrate, the liberation of acetic acid was followed using a Sargent pH stat[4]. The hydrolysis of phenyl acetate and *p*-nitrophenyl acetate was followed at pH 7.8 in a Cary Model-15 spectrophotometer at 270 and 400 nm, respectively, using the 0–0.1 absorbance slidewire. Apparent K_i values were determined from $1/v$ *vs.* $1/[S]$ plots where v = initial velocity and $[S]$ = substrate concentration.

Substrate	K_m *(mM)*	*Apparent* K_i *with* NVP^+ *(μM)*
Acetylcholine	0.20	7.5 ± 1.0
Phenyl acetate	1.2	5.5 ± 1.0
p-Nitrophenylacetate	4.7*	8 ± 2

* This value is taken from PURDIE[7].

NVP^+ with an apparent K_i of about 7 μM. This is convincing evidence that the inhibitor is acting to prevent enzyme deacylation.

This conclusion is further substantiated by the fact that NVP^+ and other styrylpyridine analogues inhibit acetylcholinesterase with non-competitive kinetics. Other workers[19,20] have found that certain quaternary ammonium ions such as *N*-methylpyridinium and tetrapropylammonium, which inhibit acetylcholinesterase non-competitively, can be shown to block the deacylation step.

α-Chymotrypsin was assayed with *p*-nitrophenyl acetate as substrate by a spectrophotometric method[17]. No inhibition of the enzyme could be detected at concentrations of NVP^+ as high as 1 mM.

Multiple inhibition studies

POTTER *et al.*[10] have reported that 10 μM $CuSO_4$ completely inhibited a rat brain preparation of choline acetyltransferase. With our caudate nucleus extract, $CuSO_4$ and $HgCl_2$ both were inhibitory (Fig. 5). The inhibitor concentration when the ordinate is zero represents the I_{50} value under the assay conditions used. The slopes of these Hill plots are about 2 for both Hg^{2+} and Cu^{2+}, which implies that two of these ions interact at each enzyme active site to produce inhibition. Dithiothreitol at 0.2 mM completely reversed the inhibition by both Cu^{2+} and Hg^{2+}, as might be expected because of the strong binding of these cations to thiols. However, 0.2 mM histidine reversed only the inhibition by Cu^{2+} and this reversal was complete at Cu^{2+} concentrations below the I_{50} value. Although both of these cations may react with several amino acid moieties, Hg^{2+} has a greater affinity for sulfhydryl groups, and Cu^{2+} at low concentrations is thought to interact preferentially with imidazole[21].

The hydrolysis of 0.5 mM acetylcholine by acetylcholinesterase from *E. electricus* also was inhibited by Cu^{2+}, with 50% inhibition evident at 20 μM. Since the cholinesterase is believed to contain one or more catalytic imidazole groups and no catalytic thiol groups[22], it is likely that Cu^{2+} inhibited this enzyme by interaction with imidazole moieties.

Fig. 5 demonstrates inhibition of choline acetyltransferase by the sulfhydryl-specific compound, allicin[5,23]. This compound was a relatively weak inhibitor of choline acetyltransferase, and the slope of 0.4 in Fig. 5 indicates that interaction of the enzyme with allicin produced only a partial inhibition[13]. It is quite possible that allicin produced configurational changes as a result of interaction with sulfhydryl groups, without necessarily binding at a strategic catalytic site. The inhibition was completely reversed when 0.2 mM dithiothreitol was added to the assay after preincubation of the enzyme and allicin at 5° for 40 min. Another sulfhydryl reagent, *N*-ethylmaleimide[24,25], produced only 4% inhibition at a concentration of 50 μM.

Each of the three inhibitors (Fig. 5) was used in enzyme assays in combination with NVP^+ at 0.65 μM, a concentration which normally produced 50% inhibition in the absence of other inhibitors. In these experiments Hg^{2+}, Cu^{2+}, or allicin was always added to the enzyme before NVP^+, and substrates were added last. In the presence of Hg^{2+} concentrations between 0.5 and 5 μM, NVP^+ inhibited any remaining activity by 50 $\pm$ 7%. This would be expected if the first inhibitor, Hg^{2+}, irreversibly converted a fraction of the enzyme to a form which was inactive and inaccessible to NVP^+. As allicin concentration was increased, an I_{50} concentration of NVP^+ exhibited a gradually decreasing effect on the inhibition. Enzyme activity decreased from 50% of the

TABLE IV

MULTIPLE INHIBITION OF CHOLINE ACETYLTRANSFERASE

The concentration of each inhibitor necessary to give 50% inhibition (I_{50}) under the assay conditions was first determined in the absence of other inhibitors. All inhibitors were then used at these I_{50} concentrations to produce the data below. Where two inhibitors were used together, NVP^+ was added to the extract after the other compound, and substrates were added last to give 50 μM [^{14}C]acetyl–CoA and 5.0 mM choline. Percent enzyme activity is based on the activity of uninhibited controls.

Inhibitor (μM)	*Activity (%)*
None	100
NVP^+ (0.65)	50
NVP^+ (0.65) and Hg^{2+} (1.5)	28
NVP^+ (0.65) and allicin (35)	29
NVP^+ (0.65) and Cu^{2+} (7)	40

uninhibited control in the presence of NVP^+ alone to 29% when 35 μM of allicin, its I_{50} concentration from Fig. 5, was initially present. If allicin inhibition was kinetically irreversible, the remaining activity after NVP^+ addition theoretically would have been 25%.

When $CuSO_4$ and NVP^+ were used together in a similar experiment, the result was quite different, as shown in Table IV. This table compares data obtained when all inhibitors were present at their usual I_{50} concentrations. In the presence of Cu^{2+}, the remaining enzyme activity appeared to be partially protected from NVP^+ inhibition. This effect is illustrated in Fig. 6, in which the percent inhibition by NVP^+ is based on the activity of the enzyme in the presence of Cu^{2+}.

No interactions between NVP^+ at 5 μM and Cu^{2+} or Hg^{2+} at 10 μM in phosphate buffer were observed spectrally, using a Cary Model-15 spectrophotometer.

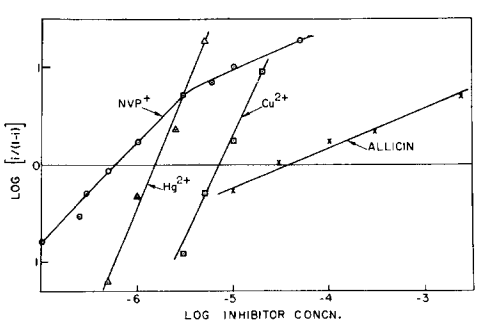


Fig. 5. Inhibition of choline acetyltransferase by several inhibitors. Acetyl–CoA concentration was 50 μM; choline, 5.0 mM. Where log $[i/(1-i)] = 0$, molar I_{50} concentrations may be determined. With NVP^+, I_{50} was essentially independent of substrate concentrations. With the other inhibitors, substrate concentrations were not varied.

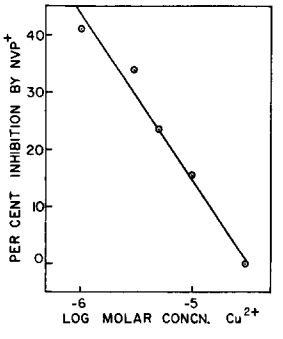


Fig. 6. Apparent protection of choline acetyltransferase activity by $CuSO_4$. NVP^+ was present at 0.65 μM, a concentration which gives 50% inhibition in the absence of Cu^{2+}. Acetyl–CoA concentration was 50 μM; choline, 5.0 mM.

TABLE V

STIMULATION OF CHOLINE ACETYLTRANSFERASE BY AMINES

50 μM [^{14}C]acetyl–CoA and 5 mM choline were used in the assay.

Compound	*Concn. (mM)*	*Relative enzyme activity*	*pK_a (ref. 21)*
None	—	1.00	—
Histamine	1.0	1.22	5.98
Imidazole	1.0	1.30	6.95
1-Methylimidazole	1.0	1.38	7.25
4-Aminomethylstilbene	0.1	1.26	

Enzyme stimulation by amines

Imidazole has been reported to stimulate the activity of choline acetyltransferase[26]. In our system, with acetyl–CoA and choline as substrates, several other organic amines were found to produce a similar effect, as indicated by the data in Table V. This additional formation of acetylcholine was strictly dependent on the presence of the enzyme extract, and no reaction of the substrate, [^{14}C]acetyl–CoA, was detected in the absence of choline. Therefore the stimulation was not caused by the enzymic or non-enzymic formation of a high-energy derivative of the amine, such as *N*-acetylimidazole[27].

After dialysis of the caudate nucleus extract against 0.07 M potassium phosphate buffer (pH 6.7), or this buffer containing 0.1 mM dithiothreitol, imidazole was no longer able to stimulate enzyme activity. However, after dialysis of the original extract against the above phosphate buffer containing 0.1 mM disodium EDTA, imidazole at 1 mM caused 37% stimulation. Dithiothreitol and EDTA both tend to stabilize enzyme activity in the absence of imidazole[4]. These observations are not readily explained. One possibility is that amines may activate one or more less active configurational forms of the enzyme by general acid–base catalysis, and that the proportion of such amine-susceptible forms may depend on dialysis conditions.

The inhibition of choline acetyltransferase by NVP^+ was apparently increased if imidazole was added first to the extract. A concentration of NVP^+ which normally produced 50% inhibition appeared to give 70% inhibition in the presence of 1 mM imidazole. A similar effect was observed when 4-aminomethylstilbene was used instead of imidazole. These amines may interact with sites of loss on extract proteins which would otherwise decrease the effective concentration of NVP^+. Alternatively, the amines may be prevented from exerting their stimulatory effects when NVP^+ is present. If this were the case, they might interact at the same enzyme site, a possibility which is consistent with the fact that 4-aminomethylstilbene is similar in structure to the inhibitory analogue of NVP^+, *N*-methyl-4-styrylpyridinium iodide[1].

In vitro interactions of styrylpyridine analogues

Using a Cary Model-14 spectrophotometer equipped with a 0–0.1 absorbance slidewire, difference spectra were obtained for NVP^+ with various compounds which might have relevance to NVP^+–enzyme interactions. The ultraviolet maxima of 5 μM NVP^+ were depressed approx. 10% in the presence of 0.8 mM cysteine, 0.4mM

dithiothreitol, or 50 μM CoA, but no wavelength shifts were observed. Similar spectral changes were found with other quaternary ammonium analogues of NVP^+ including those which are not inhibitors. In the presence of a 100-fold molar excess of histidine, tyrosine, or indole, small absorbance increases were observed in the long-wavelength region. No interaction with serine could be detected. With 0.67 M phenol in aqueous solution at pH 4 or 2.9 M imidazole at pH 7.8, the spectrum of 4 μM NVP^+ was slightly depressed and red-shifted from 377 to 387 nm. A similar shift of 13 nm was observed in 0.5 M adenine at pH 12 and a smaller shift of only 3 nm in 0.25 M guanine. When an aqueous 0.67 M phenol solution of NVP^+ was raised to pH 7, an insoluble product appeared, although either phenol or NVP^+ alone was completely soluble at the concentrations and pH used.

The pK_a of imidazole was increased by 0.11 pH units in the presence of 0.1 mM NVP^+. However, the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate was not inhibited by NVP^+.

Tyrosine, phenol, indole, sulfhydryl compounds, adenine, guanine, and unionized imidazole all may be considered charge-transfer electron donors which might be expected to form weak complexes with the pyridinium moiety of NVP^+ (refs. 28 and 29). However, the interactions with imidazole, phenol, and adenine, which were illustrated by bathochromic shifts in the presence of large excesses of the other component, are similar to shifts of NVP^+ spectra in more hydrophobic solvents such as ethanol, which are of the same order of magnitude and correspond to energy differences of about 3 kcal/mole.

A strong binding of NVP^+ and its analogues to DNA has been observed and is being further studied. This appears to involve intercalation of NVP^+ between base pairs as well as ionic interactions with DNA phosphates[46].

DISCUSSION

The Theorell–Chance mechanism tentatively suggested (Fig. 2) for choline acetyltransferase of the caudate nucleus is described by CLELAND[9] as a limiting case of the more general ordered bi-bi mechanism in which the steady-state concentration of the central complex is very low. The initial velocity data of POTTER *et al.*[10] for rat brain enzyme support a sequential mechanism, but these workers found that CoA and acetylcholine did not exhibit product inhibition. However, their experiments appear to have been done at relatively high substrate concentrations. KAITA AND GOLDBERG[30] recently have reported that with rat brain extract acetylcholine inhibition is competitive with choline and non-competitive with acetyl–CoA. SCHUBERTH[31], working with human placental choline acetyltransferase, has proposed a ping-pong mechanism, but this conclusion has recently been questioned by MORRIS AND GREWAAL[32] on the basis of their isotope-exchange experiments.

Our results with the caudate nucleus acetyltransferase suggest that, under the conditions of these experiments, acetyl–CoA and CoA exhibit similar affinities for the same enzyme form and that product inhibition by CoA is much stronger than that by acetylcholine. Several other workers[10,30] have discussed the possibility that product inhibition by acetylcholine may be involved in regulation of its synthesis *in vivo*. However, for every mole of acetylcholine synthesized, there is also produced 1 mole of CoA, which must be rapidly acetylated by an ATP-requiring phospho-

transacetylase system[33]. The possible role of CoA as a regulating factor in acetylcholine synthesis deserves further study.

The observation that the acetyl moiety does not appear to participate in binding of acetyl–CoA to choline acetyltransferase is consistent with the experiments of BERMAN *et al.*[34] who found that butyryl–CoA and benzoyl–CoA were inhibitors of the enzyme from squid head ganglia. These compounds apparently were bound to the acetyl–CoA binding site, but catalysis did not proceed.

Both choline acetyltransferase and acetylcholinesterase are enzymes which catalyze acetyl group transfer. With the former, the acetyl moiety is transferred from acetyl–CoA to choline and with the latter, from acetylcholine to water. Since NVP^+ inhibited both enzymes non-competitively (with apparent K_i values of 0.55 and 7.5 μM, respectively), it was reasonable to look for a similarity in their modes of inhibition. Our work with eel acetylcholinesterase has shown that NVP^+ inhibits the rate-determining enzyme deacetylation step. It has been suggested[17,18,35,36] that a catalytic histidine at the active center of cholinesterase and other hydrolytic enzymes is involved in enzyme deacylation. In this hypothesis the acetyl group, which is covalently bound to an enzyme serine moiety, is transferred to a catalytic imidazole group. The resulting less stable acetyl–imidazole intermediate is then hydrolyzed. The ability of imidazole itself to slowly catalyze the hydrolysis of *p*-nitrophenyl acetate[35,36] and of the acyl–serine active site model, *N,O*-diacetylserinamide[37], is evidence in support of this concept.

With choline acetyltransferase, gel–filtration experiments in which the [^{14}C]-acetyl moiety was detected in enzyme-containing fractions suggested that some kind of acetyl–enzyme complex was formed, which was diminished on addition of the second substrate, choline. The stabilization of this complex by NVP^+ might have resulted from any of the following effects: (a) NVP^+ may prevent removal of a proton from choline; (b) NVP^+ may interfere with acetyl transfer from one enzyme moiety to another or to choline; (c) NVP^+ may prevent release of acetylcholine from the enzyme. The additional possibility that NVP^+ might inhibit binding of choline is inconsistent with the non-competitive inhibition kinetics. Considering the behavior of acetylcholine in product-inhibition studies, (c) does not appear to be a likely mechanism. Steps (a) and (b) have counterparts in the acetylcholinesterase mechanism where a water molecule is substituted for choline. Imidazole has been implicated both as a general base catalyst in the removal of a proton from water or other acetyl receptor and as an enzymic moiety which is capable of accepting an acetyl group during enzyme deacylation[24,35–37].

A further indication that inhibition by NVP^+ may result from interference with a catalytic histidine on choline acetyltransferase can be derived from the multiple inhibition studies in which Cu^{2+}, which is thought to bind preferentially to imidazole groups[21] appeared to compete with NVP^+ for the same enzyme site. An alternative explanation for the protective effect is that Cu^{2+} and NVP^+ may bind to different active enzyme configurations, which may exist in equilibrium. Each inhibitor would thus stabilize the configurational form to which it binds, making a portion of the enzyme less accessible to the other inhibitor. In this latter situation the two inhibitors would not necessarily bind at the same enzyme site, but a mutual protective effect would result allosterically. The effect of Cu^{2+} in combination with NVP^+ is similar to that observed in multiple inhibition studies by BARRÓN AND SINGER[38] in which

succinate dehydrogenase was protected from heavy-metal inhibition by malonate. The multiple inhibition studies of FOLDES *et al.*[39] with inhibitors of plasma cholinesterase may also be analogous.

Reported pH optima for choline acetyltransferase from several sources, from microbial to vertebrate tissues, vary from 6.7 to 7.4 (refs. 31, 40–42). Although apparent pH optima may change with experimental conditions[31], this is the pH region in which acid–base catalysis by enzymic imidazole would be expected[21].

Choline acetyltransferase generally is referred to as a sulfhydryl enzyme in that it is inhibited by reagents such as *p*-chloromercuribenzoate, Cu^{2+}, and iodoacetate, and stabilized by cysteine[33]. However, all the sulfhydryl reagents used are relatively non-specific and may react with imidazole as well as other protein moieties[21]. The incomplete inhibition found with the more specific sulfhydryl reagent, allicin (Fig. 5) suggests that interaction of this compound with sulfhydryl groups may cause conformational changes to less active enzyme forms, but that the sulfhydryl groups involved are not necessarily catalytically active. The lack of inhibition after incubation of the enzyme with low concentrations of *N*-ethylmaleimide also supports this. Acetylcholinesterase, which is not considered a sulfhydryl enzyme, was inhibited by low concentrations of Cu^{2+}, similar to those which inhibit the acetyltransferase. This, combined with the strong evidence that imidazole is catalytically active in the cholinesterase mechanism[17,18,35–37], implies that low Cu^{2+} concentrations may inhibit by binding to imidazole rather than thiol groups.

The presence of dithiothreitol in the choline acetyltransferase system serves to stabilize the enzyme and to restore activity of an aged extract. If the acetyltransferase system is coupled to an acetyl–CoA generating phosphotransacetylase, the presence of a thiol such as dithiothreitol is necessary, since it is an absolute requirement for the latter enzyme[43].

Inhibition of choline acetyltransferase by NVP^+ was not reversed by dithiothreitol nor was the enzyme protected from NVP^+ inhibition by prior treatment with this dithiol. Although it is unlikely that binding of NVP^+ to an enzymic sulfhydryl moiety was responsible for the reversible inhibition observed with this compound, there remains the possibility that a secondary irreversible interaction of NVP^+ with a thiol group may occur. The partial irreversibility in the inhibition, which was observed on preincubation of enzyme and inhibitor at 37°, might have resulted from such an effect.

The relatively weak interactions between NVP^+ and various sulfhydryl compounds, histidine, tyrosine, and indole do not allow any conclusions regarding specificity of NVP^+ binding. The interaction of styrylpyridine analogues with choline acetyltransferase may, on the other hand, involve several amino acid moieties, the properties of which may be greatly modified by their environment in the catalytic configuration of the enzyme. That inhibition may depend on the tertiary structure of the enzyme itself has been shown for the choline acetyltransferase of *Lactobacillus plantarum*[43]. The specific nature of NVP^+-enzyme binding is further illustrated by the failure of NVP^+ to inhibit α-chymotrypsin. This enzyme, which is catalytically very similar to acetylcholinesterase and which hydrolyzes some of the same substrates[44], is believed, on the basis of recent spin label studies[45], to have a more inaccessible or buried active site region, compared with that of acetylcholinesterase.

The present studies lead us to tentatively propose that NVP^+ may block the

function of a catalytic imidazole during enzyme deacylation. Such an imidazole would not necessarily be bound directly to the inhibitor, but may be blocked because of steric or electronic effects resulting from inhibitor binding to other moieties.

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