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PHOTOISOMERIZATION OF STYRYLPYRIDINE ANALOGUES IN RELATION TO CHOLINE ACETYLTRANSFERASE AND CHOLINESTERASE INHIBITION

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SUMMARY

1. *Trans*-styrylpyridine analogues, which include the most potent known inhibitors of choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6), photoisomerize readily in solution to yield mainly the *cis* isomers. The ionized pyridinium forms are particularly photosensitive.

2. Inhibition of choline acetyltransferase is maximal with the *trans* isomer of naphthylvinylpyridinium compounds and decreases markedly with progressive isomerization to the *cis* form. The *trans* isomer of *N*-methyl-4-(1-naphthylvinyl)-pyridinium iodide shows an $[I_{50}]$ of 0.65 μM, but an $[I_{50}]$ of 2.7 μM is found if the inhibitor is exposed to indirect daylight for 15 min.

3. With acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) either isomer is inhibitory, the *cis* form being as potent as, or somewhat more potent than, the *trans* isomer.

4. Relative light sensitivities of various analogues are consistent with a stabilization of *cis* isomers by formation of intramolecular charge-transfer complexes.

INTRODUCTION

Some styrylpyridine* analogues have been reported as potent inhibitors of choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6)[1–3]. Most of these inhibitors are extremely light sensitive in solution, and certain precautions are required to obtain reproducible results in biological systems[4]. In this paper we describe the effects of photoisomerization on the inhibition of choline acetyltransferase and on acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7). Structural characteristics of the inhibitors which influence light sensitivity are discussed.

MATERIALS AND METHODS

Synthesis of the styrylpyridine analogues has been previously described[2,3]. All

* Optional nomenclature: stilbazole, phenylvinylpyridine, phenylethenylpyridine. Styrylpyridine analogues named without configurational notation are *trans* isomers.

work with the *trans* forms in solution was done either in darkness or in a room illuminated only by a 75-W General Electric pink tungsten bulb directed against a white ceiling. No isomerization was found to occur with this type of illumination. Stock solutions were prepared in ruby glass tubes or in foil-covered flasks.

Absorption spectra were determined using either a Cary Model 14 or Cary Model 15 spectrophotometer. Relative light sensitivities were estimated by first recording the spectrum of an unexposed solution in a stoppered quartz cuvette and then exposing the cuvette to light for a given length of time measured with a stopwatch. The spectrum was immediately determined again. Successive timed exposures of the solution in the cuvette were correlated with absorbance changes at a wavelength maximum of the compound. For a relatively constant light source, a 275-W Westinghouse sunlamp was used at a distance of 70 cm.

Fluorescence spectra were determined using an Aminco-Bowman spectrophotofluorometer with a Varian X–Y recorder.

Choline acetyltransferase from the calf caudate nucleus was kindly supplied to us by Dr. F. F. Foldes of Montefiore Hospital and Medical Center (Bronx, N.Y.). The preparation was a 30–40% $(NH_4)_2SO_4$ fractionation cut from a 100 000 $\times g$ aqueous supernatant and had a specific activity in our laboratory of 0.002 units/mg protein (μmoles/min per mg protein) at 37°. After 4 months at −15°, with frequent thawing at 0° and re-freezing, less than 10% loss of activity was detected.

Acetylcholinesterase from *Electrophorous electricus* was a chromatographically pure, lyophilized preparation from Worthington Biochemical Corp. with a specific activity of 1100 units/mg protein at 25°. Bovine erythrocyte acetylcholinesterase, with activity of 20 000 units per vial, was purchased from Nutritional Biochemicals Corp.

[^{14}C]Acetyl-CoA, 60 mC/mmole (radiochemical purity 95%) was purchased from New England Nuclear Corp; unlabeled acetyl-CoA (93% purity), bovine albumin (electrophoretically pure), and eserine sulfate from Mann Research Laboratories; choline chloride, reagent grade, from Merck and Co., Inc.; acetylcholine bromide from Sigma Chemical Co. Water for all solutions was glass-distilled.

Assay of choline acetyltransferase

The enzymatic transfer of the acetyl moiety from [^{14}C]acetyl-CoA to choline was followed. All manipulations before the incubation step were carried out in a 5° refrigerated room illuminated by a pink light bulb. The stock solution of extract was diluted in 0.07 M potassium phosphate buffer (pH 6.7) containing 0.05% bovine plasma albumin, to give an enzyme concentration which would yield 0.25 nM or less of acetylcholine after the incubation, so that not more than 10% of the [^{14}C]acetyl-CoA was consumed. Using Dispo capillary pipets (Scientific Products), 20 μl of diluted extract was added to either 10 μl of potassium phosphate buffer or 10 μl of inhibitor dissolved in the buffer. Then 20 μl of substrate mix was added to give a total volume of 50 μl. The mixture was incubated in darkness at 37° for 10 min. In a typical assay, components and concentrations were as follows: [^{14}C]acetyl-CoA, 50 μM (4 mC/mmole); choline, 5 mM; bovine plasma albumin, 0.05%; NaCl, 0.30 M; eserine, 0.2 mM; potassium phosphate, 0.07 M; pH 6.7. Optional components, which tend to stabilize enzymatic activity, were 40 μM dithiothreitol and 80 μM EDTA. The reaction was stopped by plunging the assay tubes into ice water, adding 20 μg unlabeled acetyl-

choline to each tube, and heating for 1 min in a boiling-water bath, as recommended by SCHRIER AND SHUSTER[5].

Electrophoretic separation of [^{14}C]acetylcholine was carried out by the method of POTTER AND MURPHY[6]. The unlabeled carrier acetylcholine was located on the dried strips by holding them in iodine vapor to allow formation of a transient iodine complex. Scintillation counting was done in either a Nuclear Chicago Mark I or a Packard Tri-Carb scintillation spectrometer. In toluene scintillation fluid containing 5.0 g/l of 2,5-diphenyloxazole (Packard Instrument Co.), counting efficiency for ^{14}C on the Whatman No. 1 paper electrophoresis strips was 54%.

All assays were done in duplicate or triplicate, with precision of $\pm$ 5%. Controls containing no enzyme were included in every set of experiments to provide a reagent blank, which usually involved a correction of about 10 counts/min.

Assay for acetylcholinesterase

Acetic acid released during hydrolysis of acetylcholine was automatically titrated with 5 mM NaOH, using a Sargent pH stat. Concentration of acetylcholine was varied from 0.1 to 1 mM. NaCl, 0.1 M, and $MgCl_2$, 0.02 M, were present in the assay medium. The reaction was started by adding 0.2 units of enzyme. The lyophilized eel enzyme was diluted to 10 units/ml in 0.02 M $MgCl_2$ containing 0.005% bovine plasma albumin. After overnight storage at 4°, this enzyme solution gave reproducible results and remained stable at 4° for at least a week.

pK_a determinations

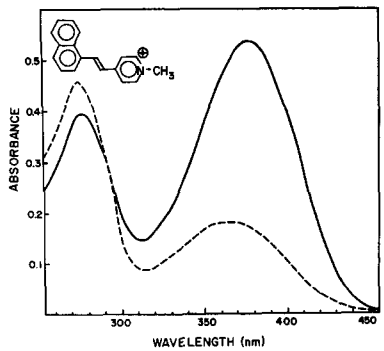
pK_a determinations of amines were done by potentiometric titration at 25°, using a Beckman Expandomatic pH meter. The relatively insoluble amines were dissolved in absolute ethanol, and water was added to yield a concentration of 0.1 mM with 3% ethanol. Portions of 50 ml were titrated with 0.1 M HCl or NaOH, using a Hamilton microsyringe.

RESULTS

Evidence for trans to cis isomerization

Absorption spectra of styrylpyridines are generally similar to those reported for stilbenes[7,8] in that as wavelength is decreased, three distinct maxima are observed, referred to as A, B, and C bands. However, with styrylpyridines these bands are shifted to much longer wavelengths. For example, the A band of stilbene occurs at 294 nm, that of *N*-methyl-4-styrylpyridinium iodide at 342 nm, and that of *N*-methyl-4-(1-naphthylvinyl)pyridinium iodide at 377 nm. The Beer–Lambert law was obeyed at the concentrations studied ($< 50\ \mu$M). The iodide anion did not appear to influence spectra, since solutions of hydrochloride salts of the free bases, for example 4-(1-naphthylvinyl)pyridine below its pK_a value, exhibit spectra very similar to those of the corresponding methiodides.

The spectral changes which occur on light exposure are illustrated in Figure 1 for N-methyl-4(1-naphthylvinyl)pyridinium iodide. Spectra were not affected by the Cary light source during assay. After 10 min exposure to indirect sunlight in a quartz cuvette a photo-stable equilibrium was reached, after which further light exposure up to 1 h caused no spectral change. The A band was markedly reduced in intensity



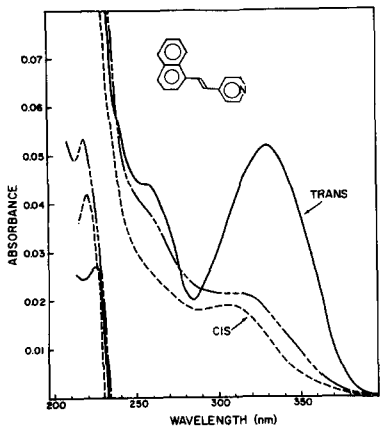


Fig. 1. Effect of light on *N*-methyl-4(naphthylvinyl)pyridinium iodide. The spectrum of an aqueous solution at 23 μM (pH 6) was recorded with a Cary Model 14 spectrophotometer before (———) and after (— — —) exposure to indirect sunlight for 10 min.

Fig. 2. Comparison of spectra of *cis*-4(1-naphthylvinyl)pyridine with the *trans* isomer, before and after light exposure. — — —, *cis*; ———, *trans*; – – ——, *trans* after light exposure for 30 min. The *cis* form did not change with light exposure. Solutions were 2.8 μM (pH7) in 1 mM potassium phosphate. A Cary Model 14 spectrophotometer was equipped with a 0 to 0.1 absorbance slidewire.

and blue-shifted from 377 to 365 nm. Both the B and C (not shown) bands were more intense and slightly blue-shifted. These changes are qualitatively similar to spectral changes which occur when *trans*-stilbene is photoisomerized[7–9], but appear to take place more readily. Similar spectral changes on light exposure were found with all styrylpyridine analogues. Naphthyl derivatives were approx. 5 times as light sensitive as corresponding phenyl derivatives.

Fig. 2 shows the spectra of both *trans*- and *cis*-4-(1-naphthylvinyl)pyridine at pH 7, where it exists in the essentially unionized form ($pK_a = 5.5$). The third spectrum shows the *trans* form after light exposure for 30 minutes. It appears that 80–90% of the *trans* isomer has been changed to the *cis* form in the light-exposed solution. This is in contrast to studies with stilbenes[9], which indicate that only 30–35% conversion from *trans* to *cis* form occurs before a photo-stable equilibrium is reached.

Further evidence for *trans* to *cis* photoisomerization of the styrylpyridines was obtained from fluorescence spectra. The *trans* form of *N*-methyl-4-(1-naphthylvinyl)-pyridinium iodide fluoresces strongly with an excitation maximum at 375 nm and emission at 518 nm. Both emission and excitation spectra decrease rapidly as a result of irradiation by the strong xenon light source in the instrument. At an exciting wavelength of 375 nm, intensity of emission at 520 nm was followed as a function of time. Intensity decreased initially in exponential fashion with a half-life of about 30 sec. After several minutes an equilibrium state was reached, with the solution exhibiting about 20% of the original fluorescence intensity.

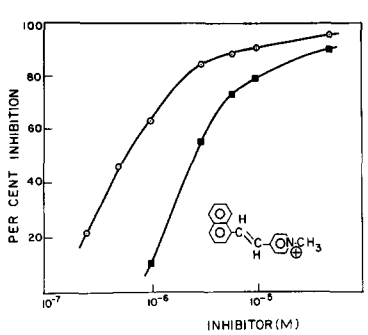
LEWIS *et al.*[9] have reported that *trans*-stilbene is highly fluorescent, while *cis*-stilbene is not fluorescent. The fluorescence remaining in the light-exposed solutions of our stilbazole analogues probably is caused mainly by *trans* isomer which exists

in photostable equilibrium with the *cis* form. However, it is possible that the *cis* forms of styrylpyridines may possess some fluorescence of their own.

An interesting point is that excitation at the long wavelength absorption maximum (A band) is responsible for fluorescence and is of sufficient energy to produce rapid isomerization. Other styrylpyridine analogues behaved in a similar manner. However when the inter-annular bond was acetylenic rather than ethylenic, the compound was not light sensitive and exhibited only weak fluorescence.

Effect of photoisomerization on enzyme inhibitions

Figs. 3 and 4 illustrate the effect of *trans* to *cis* isomerization with two of the most potent inhibitors of choline acetyltransferase from calf caudate nucleus. Since



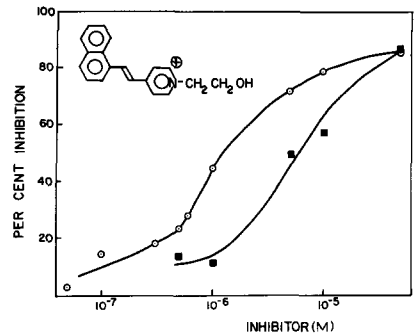


Fig. 3. Inhibition of choline acetyltransferase of calf caudate nucleus by *N*-methyl-4-(1-naphthylvinyl)pyridinium iodide. ○, *trans*; ■, photo-stable inhibitor.

Fig. 4. Inhibition of choline acetyltransferase of calf caudate nucleus by *N*-hydroxyethyl-4(1-naphthylvinyl)pyridinium iodide. ○, *trans*; ■, photo-stable inhibitor.

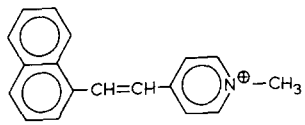
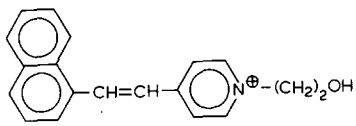
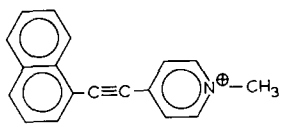
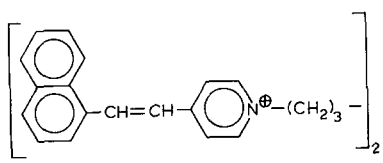
$[I_{50}]$ values obtained with the styrylpyridine analogues were essentially independent of the concentration of either substrate (non-competitive inhibition in which plots of reciprocal of initial velocity *vs.* reciprocal of variable substrate concentration intersect on the abscissa)[10], these values may provide a suitable indication of relative inhibitory potencies under the assay conditions used. *N*-Methyl-4-(1-naphthylvinyl)-pyridinium iodide (Fig. 3) represents a highly potent inhibitor; however, the *N*-(hydroxyethyl)-derivative shown in Fig. 4 also is of interest in that it is considerably more water soluble, and therefore may be more appropriate for pharmacological studies. With both compounds isomerization to the *cis* isomer raises the $[I_{50}]$ value, from 0.65 to 2.7 μM for *N*-methyl-4-(1-naphthylvinyl)pyridinium iodide and from 1.2 to 6 μM for the *N*-(hydroxyethyl)-analogue. It was estimated spectrophotometrically that the photo-stable equilibrium solutions may still contain as much as 20% of the *trans* isomer. Therefore, choline acetyltransferase appears to be significantly inhibited only by the *trans* isomer. This is compatible with the inactivity reported for a pure *cis* isomer[2].

With acetylcholinesterase the effect of isomerization is quite different. Both isomers were inhibitory, the *cis* being at least as potent as the *trans*, as judged from apparent K_i values determined from slopes of reciprocal plots. The overall mechanism of inhibition was noncompetitive with both isomers, but reciprocal plots for the *cis*

TABLE I

EFFECT OF PHOTOISOMERIZATION ON ENZYME INHIBITIONS

Inhibitor solutions at 5-fold the desired assay concn. were prepared from a single stock solution in ruby-glass tubes. A portion of each was exposed to indirect sunlight for 15 min before use in the assays, as described in MATERIALS AND METHODS. [I_{50}]values with choline acetyltransferase were obtained from plots similar to Figs. 3 and 4. Apparent K_i values for acetylcholinesterase were obtained by the customary LINEWEAVER–BURK plotting procedures[11], as described in the text.

Inhibitor	*Acetyltransferase* (I_{50}) (μM)		*Esterase* (eel)* K_i (μM)		*Esterase* (erythrocyte)* K_i (μM)	
	dark	*light*	*dark*	*light*	*dark*	*light*
(1) 	0.65	2.7	7.5	6.8	8.3	3.3
(2) 	1.2	6.0	11	13		
(3) 	1.7	1.7	7.5	7.5		
(4) 					1.7	0.78

* These results show considerably greater esterase inhibitory activities than previously reported[1–3] for inhibition of acetylcholinesterase in crude extract of rat brain.

isomers intersected above, rather than on, the abscissa. Table I compares results obtained with choline acetyltransferase from calf caudate nucleus and acetylcholinesterases from electric eel and bovine erythrocytes. An acetylenic analogue, which cannot photoisomerize, was included in the table as a control.

The K_i values in Table I are apparent K_i values determined from the ratio of slopes of reciprocal plots in the presence and absence of inhibitor.

$$\frac{\text{slope } (+ I)}{\text{slope } (- I)} = 1 + \frac{[I]}{K_i}$$

Since the inhibitors may combine with more than one enzyme form, these apparent K_i values do not represent true enzyme–inhibitor dissociation constants,

but are used to indicate relative effectiveness of the inhibitors under the assay conditions.

Photoisomerization related to electronic structure

The relative light sensitivities of styrylpyridine analogues appear to be related to the electron donor–acceptor character of the aryl and pyrido-ring moieties joined by the double bond. 4-(1-Naphthylvinyl)pyridine behaves in a manner similar to the corresponding quaternary derivative in the pH region below its pK_a value of 5.5. The naphthyl moiety is an electron donor and the protonated pyridine moiety is an acceptor[12]. However, at pH 8, where the unionized pyridine moiety acts as an electron donor, this compound exhibits only slight photosensitivity. Fig. 5 illustrates this

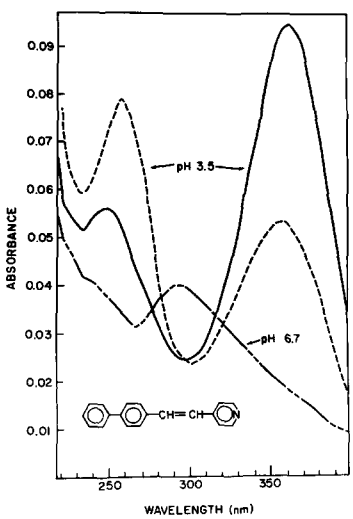


Fig. 5. Effect of pH on photosensitivity. Aqueous solutions of the compound (pK_a = 4) at 5 μM at pH's 6.7 and 3.5 were exposed to indirect sunlight for 2 min. Spectra were determined with a Cary Model 15 spectrophotometer before (——) and after (- - - -) light exposure. The spectrum at pH 6.7 did not change.

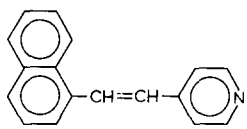
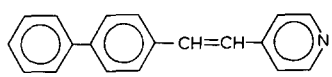
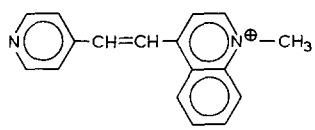
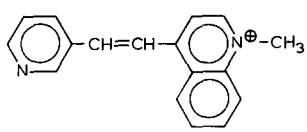
same effect for another analogue, *trans*-4-(*p*-biphenylylvinyl)pyridine. The pK_a of this compound is 4.0, and therefore the donor–acceptor form at pH 3.5 isomerized rapidly with light exposure, but the donor–donor form at pH 6.7 appeared to be photo-stable under our conditions. The importance of a donor–acceptor electronic structure for photosensitivity is further substantiated by the properties of Compounds 3 and 4 in Table II. In these examples, the quinolinium system serves as electron acceptor and the pyridine moiety can act as donor in the unionized state or as acceptor in the protonated condition at low pH. Here, as the hypothesis would require, photosensitivity is increased in basic solution.

The relative light sensitivities of a series of stilbazoles substituted *para* on the phenyl ring are presented in Table III. It is clear that no relation exists between light sensitivity and the Hammett sigma parameter[13]. These results do, however, correlate roughly with the estimated resonance effects of the particular substituents on a

TABLE II

TRANS TO *CIS* PHOTOISOMERIZATION RELATED TO DONOR–ACCEPTOR STRUCTURE

Absorbance changes were measured, as described in MATERIALS AND METHODS, at concentrations of 5–50 μM. Photosensitivity indicates response to a 30-sec exposure of the solution to a 250-W sunlamp: +, >10% drop in absorbance at λ_{max} shown for each species; sl, <2%; —, no change observed. pK_a values of compounds were determined by potentiometric titration for the first two compounds and estimated from spectral changes for the last two.

Compound		*pKa*	*pH of experiment*	λ_{max} *(nm)*	*Photosensitivity*
(1a)		5.5	3.5	370	+
(1b)			8.0	330	sl
(2a)		4.0	3.5	360	+
(2b)			6.7	290	—
(3a)		4–6	2.0	325	—
(3b)			8.0	355	+
(4a)		4–5	2.0	320	—
(4b)			8.0	365	+

conjugated system. The resonance values, which are derived from the Hammett parameters, are thought to express the contribution of electron withdrawing or releasing tendency by the substituent[14]. Both Cl and Br are substituents which contribute electronegativity to an aromatic ring, while CN and NO_2 tend to withdraw electrons from the ring. Consequently the first two substituents would strengthen electron donor properties of the styrylpyridine donor aryl moiety, but the latter two substituents would tend to weaken them.

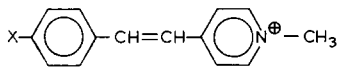
DISCUSSION

Intramolecular charge-transfer complexes consisting of an aromatic donor and an aromatic acceptor group joined by a $-CH_2CH_2-$ bridge have been studied by SCHIFRIN[15–17]. The two planar moieties in such compounds are 3.4 Å apart at their centers and deviate 39° from a parallel orientation. If a styrylpyridine analogue consists of a pyridinium moiety joined to an aromatic electron donor moiety through

TABLE III

RELATIVE LIGHT SENSITIVITIES

Change in absorbance at the long wavelength maximum of each compound was plotted *vs.* time of light exposure to a 250-W sunlamp. Initial slopes were determined and are expressed relative to that of the parent compound as s_x/s_p. R is the resonance contribution of the substituent as expressed by TAFT[14].



X	s_x/s_p	*Hammett*[13] *σ parameter*	R
(1) Br	1.6	0.23	−0.22
(2) Cl	1.1	0.23	−0.24
(3) H	1.0	0	0
(4) CN	0.67	0.63	+0.07
(5) NO_2	0.62	0.78	+0.15

an exocyclic double bond, the *cis* isomer may be expected to be partly stabilized by intramolecular charge-transfer. The distances between the two ring moieties and the angles of deviation from parallelism would be expected to vary with the intramolecular charge-transfer energy of specific derivatives. Analogy with bond angles in a planar five-atom ring would suggest the order of 36° deviation from parallelism of the bonds linking the donor and the acceptor rings to the exocyclic ethylenic carbon atoms in a *cis* configuration. The formation of such a stabilized *cis* complex would provide an explanation for the enhanced photosensitivity of *trans*-styrylpyridinium compounds in comparison with *trans*-stilbenes.

A recent report[18] describes water addition to the double bond of 1,2-bis-(pyridinium)ethylenes exposed to light. A similar reaction proceeds in good yield with *N*-methyl-4-(1-naphthylvinyl)pyridinium iodide after a 2-h exposure to direct sunlight[3]. Such addition products were not detected after the brief exposures necessary for photoisomerization. However, since definite isosbestic points were not obtained in spectra during isomerization, it is possible that small amounts of addition products may have been formed.

There appears to be no direct relation between ability of an inhibitor to isomerize and its inhibitory potency toward choline acetyltransferase. For example, Compound 3 in Table I is a potent inhibitor, but cannot isomerize since it has a linear structure. In contrast Compound 3b in Table II isomerizes readily, but is a relatively poor inhibitor ($[I_{50}] = 2 \cdot 10^{-4}$). However, the donor–acceptor character of the *trans* structures may influence both isomerization and inhibitory properties. For strong inhibition of choline acetyltransferase, the compound must be coplanar, and the electron donor moiety must be an aromatic π-cloud donor such as naphthyl or phenyl[2,3]. A heterocyclic donor (*cf.* 3b and 4b in Table II) apparently allows a localization of charge which impairs inhibitory potency. For photoisomerization, either type of donor structure confers photosensitivity and coplanarity of the whole molecule is not a requirement (*cf.* 2a in Table II).

The *trans* isomers inhibited both choline acetyltransferase and acetylcholinesterase, but isomerization to *cis* forms produced different effects with the two enzyme

systems. *Cis* isomers were no longer able to inhibit the acetyltransferase, while they still inhibited the esterase, but with a modified mechanism. These observations indicate the necessity of taking precautions to control photoisomerization in all studies in which this class of inhibitors is used.

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REFERENCES

1 J. C. Smith, C. J. Cavallito and F. F. Foldes, *Biochem. Pharmacol.*, 16 (1967) 2438.
2 C. J. Cavallito, H. S. Yun, J. C. Smith and F. F. Foldes, *J. Med. Chem.*, 12 (1969) 134.
3 C. J. Cavallito, H. S. Yun, T. Kaplan, J. C. Smith and F. F. Foldes, *J. Med. Chem.*, 13 (1970) 221.
4 H. L. White and C. J. Cavallito, *Federation Proc.*, 28 (1969) 291.
5 B. K. Schrier and L. Shuster, *J. Neurochem.*, 14 (1967) 977.
6 L. T. Potter and W. Murphy, *Biochem. Pharmacol.*, 16 (1967) 1386.
7 H. Suzuki, *Electronic Absorption Spectra and Geometry of Organic Molecules*, Academic Press, New York, 1967, p. 306.
8 H. H. Jaffé and M. Orchin, *Theory and Applications of Ultraviolet Spectroscopy*, Wiley, New York, 1962, p. 276.
9 G. N. Lewis, T. T. Magel and D. Lipkin, *J. Am. Chem. Soc.*, 62 (1940) 2973.
10 H. L. White and C. J. Cavallito, *Biochim. Biophys. Acta*, in the press.
11 H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658.
12 S. Shifrin, in B. Pullman, *Molecular Associations in Biology*, Academic Press, New York 1968, p. 323.
13 E. S. Gould, *Mechanism and Structure in Organic Chemistry*, Holt, New York, 1959, p. 221.
14 R. W. Taft, in M. S. Newman, *Steric Effects in Organic Chemistry*, Wiley, New York, 1956, p. 595.
15 S. Shifrin, *Biochemistry*, 3 (1964) 829.
16 S. Shifrin, *Biochim. Biophys. Acta*, 96 (1965) 173.
17 B. R. Baker, *Design of Active-Site-Directed Irreversible Enzyme Inhibitors*, Wiley, New York, 1967, p. 33.
18 M. T. McCall and D. G. Whitten, *J. Am. Chem. Soc.*, 91 (1969) 5681.