H_2O–NaBr in fine needles, mp 308° dec. *Anal.* ($C_{31}H_{22}Cl$-$N_5O \cdot 2HBr$) C, H, N.

7, R = NO_2.—Scarlet needles were obtained from EtOH–H_2O–HCl, mp > 360°. *Anal.* ($C_{31}H_{22}N_6O_3 \cdot 2HCl$) C, H, N, Cl. Attempted drying of this salt at elevated temp *in vacuo* caused loss of HCl and analyses showing a Cl^- content between 1 and 2 moles of Cl were obtained for samples so dried. For analysis a sample was dried at room temp *in vacuo*.

2-Amino-4-[*p*-(*p*-nitrophenylcarbamoyl)anilino]-6-methylpyrimidine.—4-(*p*-Nitrophenylcarbamoyl)aniline (2.57 g) was dissolved in hot 2-ethoxyethanol (60 ml) and the soln cooled to 60°. 2-Amino-4-chloro-6-methylpyrimidine (1.58 g) was added, the mixt was boiled till homogeneous, concd HCl (0.95 ml) was added, and the clear soln was heated on the water bath for 0.5 hr. After a few minutes a product started to crystallize. After thorough cooling the chunky pale yellow prisms of the hydrochloride were collected, suspended in EtOH (15 ml), and stirred with concd NH_3 (10 ml) for 0.5 hr. The deep yellow base sepd from DMF–H_2O–NH_3 as fine needles, (3.2 g), mp 334–335°. *Anal.* (C_{18}-$H_{16}N_6O_3$) C, H, N.

2-Amino-4-[*p*-(*p*-aminophenylcarbamoyl)anilino]-6-methylpyrimidine was prepd by Fe^{19} reduction of the preceding nitro compound in 65% DMF–H_2O. The pure compound sepd from EtOH–H_2O–NH_3 as colorless needles, mp 280–281°. *Anal.* ($C_{18}H_{18}N_6O$) C, H, N.

8.—A sample of the above primary amine (0.98 g) and 9-chloroacridine (0.64 g) were dissolved in 65% EtOH (80 ml) by boiling. HCl (12 *N*, 0.52 ml) was added to the clear soln, yellow crystals of product·2HCl started to sep shortly afterwards. The reaction mixt was thoroughly chilled after a further 0.5-hr heating on the water bath. The sepd crystals were recrystd from DMF–H_2O–HCl–NaCl. The fine yellow needles of dihydrochloride (1.48 g) had mp 358° dec. *Anal.* ($C_{31}H_{25}N_7O \cdot 2HCl$) C, H, N, Cl.

Biological Testing.—The routine screening test consists of ip inoculation of 10^5 L1210 cells into 18.5–22.5 g of C_3H–DBA_2 F_1 hybrids on day 1 with drug treatment initiated 24 hr later and continued for 5 days. All dosage was in 0.2-ml vol in H_2O suspension. Groups of 6 animals/dose level were used with one control group for every 5 tests. The wt change column in Table V records the difference between initial wt and that at day 8 for survivors. The number of animals surviving as long or longer than controls is listed under survivors. Doses have been rounded off to two significant figures. Details of testing of inactive compds have not been given.

TABLE V

Drug	Dose, mg/kg per day	Survivors	Wt change	Average survival, days: Treated	Control	T/C, %
5, R = CH_3, as dibromide	150	4	−4.2	15.9	10.2	156
	100	6	−1.8	19.2	10.2	192
	67	6	−0.2	18.2	10.2	178
	44	6	+0.6	15.1	10.2	148
	29	6	+1.1	13.7	10.4	132
	20	6	+3.2	12.8	10.4	
6, R = CH_3 as bis(*p*-toluenesulfonate)	75	4	−3.9	14.4	10.1	142[a]
	50	6	−1.8	29.5	10.1	292[b]
	33	6	−2.2	21.7	10.1	215
	22	6	−0.8	18.3	10.1	181
	15	6	+0.3	15.8	10.1	156
	10	6	−0.9	14.0	9.8	143
	6.7	6	+3.0	12.2	9.8	125
6, R = H, as monochloride	225	6	−3.8	14.5	10.2	142
	150	6	+0.8	17.8	10.6	168
	100	6	+2.9	17.2	9.8	172
	67	6	+2.4	13.4	10.1	135
	44	6	+3.5	12.7	10.4	132
7, R = NO_2, as dihydrochloride	60	5	−2.4	14.8	10.4	142
	40	6	−1.2	16.8	10.4	162
	27	6	+0.8	14.0	10.2	143
	18	6	+4.0	13.1	10.1	132
	12	6	+3.8	12.3	10.1	122
8, as dihydrochloride	350	6	−5.2	14.4	9.8	141
	230	6	−2.8	16.5	10.2	168
	155	6	+0.2	14.8	10.4	154
	100	6	+2.4	13.7	10.4	143
	70	6	+4.2	12.3	10.1	124

[a] Not including one 100-day survivor. [b] Not including two 100-day survivors.

Acknowledgments. We are grateful to Miss Lois Armiger and her assistants for technical assistance in performing the many biological tests. This work was supported by the Auckland Division, Cancer Society of New Zealand (Inc.).

Irreversible Enzyme Inhibitors. 181.[1,2] Inhibition of Brain Choline Acetyltransferase by Derivatives of 4-Stilbazole

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Forty-three analogs of 4-stilbazole were synthesized and evaluated as inhibitors of choline acetyltransferase from rabbit brain. The most active inhibitor found for the acetyltransferase was 3′,4′-dichloro-4-stilbazole (**23**) which complexed to the enzyme 910 times more effectively than the substrate choline and 230 times more effectively than 4-stilbazole. Other highly effective derivatives of choline acetyltransferase were the 3′-Cl (**11**), 3′-CH_3 (**12**), and 3′-CH_3O (**13**) derivatives of 4-stilbazole which complexed 80- to 130-fold more effectively to the enzyme than choline. Compounds **11**–**13** and **23** were poor inhibitors of the AChE from rabbit brain; for example, **11** was complexed 130-fold more tightly to choline acetyltransferase than AChE.

The major enzyme in nerve endings involved in nerve impulses is acetylcholinesterase (AChE).[3] The order of events appears to be (a) active transport of choline through the nerve membrane,[4] (b) acetylation of choline to ACh with acetyl-CoA mediated by choline acetyltransferase,[5] (c) hydrolysis of ACh to choline and acetate by AChE during the nerve impulse,[3] and (d) reacetylation of CoA.[6] A tremendous amount of

(1) This work was generously supported by Grants CA-08695 and NS-09544 from the U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker and M. Cory, *J. Med. Chem.*, **14**, 119 (1971).

(3) L. S. Goodman and A. Gilman, "The Pharmacological Basis of Therapeutics," Macmillan Co., New York, N. Y., 1960, p 422.

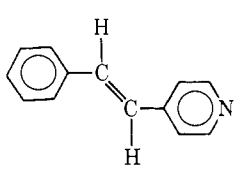
(4) L. T. Potter in "The Interaction of Drugs and Subcellular Components in Animal Cells," P. N. Campbell, Ed., J. and A. Churchill, Ltd., London, 1968, p 293.

(5) (a) Reference 3, p 408; (b) D. Nachmansohn in "Cholinesterases and Anticholinesterases," G. B. Koelle, Ed., Springer-Verlag, Berlin, 1963, p 41.

(6) D. Nachmansohn in "Handbuch der Experimentellen Pharmakologie," G. B. Koelle, Ed., Vol. 15, Springer-Verlag, Berlin, 1963, p 40.

research has been done on inhibition of AChE,[7] but practically nothing of significant use was done on inhibition of choline acetyltransferase[5] until 1967.

In 1967 Cavallito, *et al.*, reported the first[8] of a series of exciting papers[9-12] on inhibition of choline acetyltransferase by derivatives of 4-stilbazole (**1**); when the enzyme was assayed with 5 m*M* choline, **1** showed



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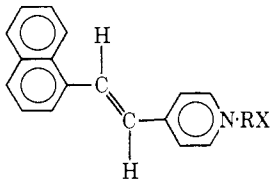

2, RX = HCl
3, RX = CH_3I

50% inhibition at 0.6 m*M*.[8] Activity was enhanced 24-fold with the 1-naphthyl derivative **2**; this activity could be further enhanced 53-fold by quaternization of **2** to **3**, that is **3** had I_{50} = 0.47 μM.[10] Replacement of the phenyl moiety of **1** by 5-indanyl, 4-biphenylyl, or 4-pyridyl led to decreased binding to the enzyme.[9]

If a choline acetyltransferase inhibitor is to be effective on a brain enzyme, at least three criteria must be met: (a) the compound should be highly potent (I_{50} < 10 μM), (b) it should be able to pass the blood–brain barrier and penetrate other membranes, (c) it should be a relatively ineffective inibitor of AChE. For example, **2** was >100 times as potent an inhibitor of choline acetyltransferase than the cholinesterase, whereas **3** was about 400 times as potent against the acetyltransferase. Furthermore, **2** could pass the appropriate membranes to the brain since it could enhance the behavioral-stimulating effects of amphetamine in trained rats, but **3** showed no activity,[13] indicating that **3** with its cationic group could not cross the appropriate membranes.

In spite of the difficulty of membrane passage with quaternary compounds of type **3**, Cavallito, *et al.*, performed the remainder of their studies with quaternary compounds;[8-10] the latter type such as **3** certainly have two advantages over compounds of type **2** in that type **3** are more water soluble and are more potent inhibitors of choline acetyltransferase in broken cell systems where the membrane transport problem has been deleted.[8-10]

Since Cavallito, *et al.*,[8-10] assayed only 5 compounds without a quaternary group, we decided to make an extended study of 4-stilbazole (**1**) analogs without a quaternary group with the expectation that each 10-fold increase in potency would approximately compensate for a 10-fold loss in solubility. The results are the subject of this paper.

Enzyme Results.—Choline acetyltransferase and AChE were isolated from rabbit brain acetone powder by the method of Potter, *et al.*[14] Choline acetyltransferase was assayed with 1 m*M* choline bromide and 0.05 m*M* [^{14}C]acetyl-CoA by suitable modification of the method of McCaman and Hunt[15] as described in the Experimental Section. AChE was assayed by modification of the method of Potter[16] using 1 m*M* [^{14}C]-ACh·Cl$^-$, as described in the Experimental Section.

The reported inhibition of choline acetyltransferase by 4-stilbazole (**1**)[8,9] was confirmed; **1** had I_{50} = 470 μM (Table I) and was complexed to the enzyme about twice as strongly as choline under our assay conditions. No inhibition of AChE was shown by **1**, although solubility was a limiting factor. That the benzene ring was essential for inhibition of choline acetyltransferase was shown by the lack of inhibition by 3000 μM 4-vinylpyridine.

A study of substituent effects on binding of the Ph ring of 4-stilbazole (**1**) to choline acetyltransferase was then made. Cavallito, *et al.*,[8,9] had studied only four analogs of the Ph moiety of 4-stilbazole where the pyridine N was not quaternized; these compounds had the Ph moiety of **1** replaced by α-naphthyl (**2**), 5-indanyl, 4-biphenylyl, and 4-pyridyl. The most active compound was **2** with I_{50} = 25 μM and $[S/I]_{0.5}$ = 200.

The effects of single small substituents were first investigated. Introduction of 4-Cl (**5**) or 4-Me (**6**) gave 3-fold and 9-fold increments in binding to choline acetyltransferase. Introduction of the more polar CH_3O (**7**), NH_2 (**8**), AcNH (**9**), or NO_2 (**10**) led to a decrease in binding. Thus the effect of the 4-Cl and 4-CH_3 can be accounted for by hydrophobic bonding. Introduction of a 2-Cl (**17**) or 2-CH_3O (**18**) led to no change in binding.

The most dramatic effects on binding were seen with 3 substituents. The 3-Cl (**11**) gave a 60-fold increment in binding and the 3-Me (**12**) and CH_3O (**13**) a 40-fold increment in brinding. Since the maximum increment in hydrophobic bonding that can be expected from Cl, CH_3, or CH_3O (π = 0.71, 0.56, −0.02, respectively)[17] from hydrophobic bonding is only 10-fold,[18] other modes of binding such as electronic effects, point donor–acceptor binding, or conformational change in the enzyme must be playing a role.[19] As we shall show later, these 3 substituents all bind to the same locus on the enzyme, that is, the benzene ring is not turned over so that there are two possible loci for binding a meta substituent; since CH_2 cannot bind by donor–acceptor point complexing, it follows that the 3 substituents have a pronounced electronic effect on binding by the benzene ring of the 4-stilbazole system. Although the NH_2 group of **14** is strongly electron releasing, it is so polar (π = −1.23)[17] that it would be repulsed from a hydrophobic locus. The polar and electron-withdrawing CN (**15**) and NO_2 (**16**) groups are detrimental to binding to choline acetyltransferase supporting the argument that nonpolar, electron-releasing groups give the best enhancement in binding at the meta position; these argu-

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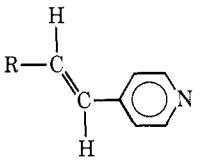
TABLE I

INHIBITION[a] OF CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE FROM RABBIT BRAIN BY



		Choline acetyltransferase[b]				Acetylcholinesterase[c]			
No.	R	Inhib, μM	% inhibn	I_{50},[d] μM	$[S/I]_{0.5}$[e]	Inhib, μM	% inhibn	I_{50},[d] μM	$[S/I]_{0.5}$[e]
1[f]	C_6H_5			470	2.1	125[g]	0	>500	<2
4[h]	H	3000	0	>12,000	<0.08				
5	4-ClC_6H_4			170	6.1	250[g]	20		
6	4-$CH_3C_6H_4$			55	18	30[g]	11		
7	4-$CH_3OC_6H_4$	500	0	>2000		62[g]	0		
8	4-$NH_2C_6H_4$	62[g]	0	>250		31[g]	0		
9	4-$AcNHC_6H_4$	250	0	>1000		125[g]	0		
10	4-$NO_2C_6H_4$	300[g]	14			37[g]	16		
11	3-ClC_6H_4			7.8	130	500[g]	33	1000[i]	1.0
12	3-$CH_3C_6H_4$			12	83	60[g]	0	>240	<4
13	3-$CH_3OC_6H_4$			13	77	250[g]	20	1000[i]	1.0
14	3-$NH_2C_6H_4$	1000	37	1700[i]	0.59	250[g]	10	>1000	<1
15	3-C_6H_4CN	500	19	~2500				100	10
16	3-$NO_2C_6H_4$	500	13	>1500	<0.7			180	5.6
17	2-ClC_6H_4			640	1.6			610	1.6
18	2-$CH_3OC_6H_4$			500	2	500[g]	0	>2000	<0.5
19[h]	4-Pyridyl	1000	0	>4000	<0.25			1700	0.59
20[h]	3-Pyridyl	500[g]	0	>2000	<0.5	500[g]	36	900[i]	1.1
21	2-Pyridyl	1000	9	>4000	<0.25	500[g]	31	1100	0.91
22	2,4-$Cl_2C_6H_3$	500[g]	0	>2000	<0.5	62[g]	16	>300	<3
23	3,4-$Cl_2C_6H_3$			1.1	910	30[g]	0	>120	<8
24	β-Naphthyl			52	19	50[g]	0	>200	<5
25	3,4-Methylenedioxyphenyl	500[g]	30	1200[i]	0.83				
26	3,4,5-$(CH_3O)_3C_6H_2$	250[g]	0			31[g]	12	>120	<8
27	4-$EtOC_6H_4$	250[g]	16	>1000	<1				
28	4-$C_6H_5CH_2OC_6H_4$	50[g]	0	>200	<5	3[g]	0	>12	
29	4-$C_6H_5(CH_2)_3OC_6H_4$	50	17	>200	<5	20[g]	0	>80	
30	4-EtC_6H_4			350	2.9	63[g]	12	>240	<4
31	4-$C_6H_5CH_2C_6H_4$	200[g]	0	>800	<1.2				
32	4-$C_6H_5CH{=}CHC_6H_4$	100[g]	0	>400	<2.5				
33	4-$C_6H_5(CH_2)_4C_6H_4$	100[g]	18	>400	<2.5				
34	3-$EtOC_6H_4$			240	4.2	500[g]	0	>2000	<0.5
35	3-(*i*-$C_5H_{11}O)C_6H_4$	167[g]	0	>670	<1.5				
36	3-$C_6H_5CH_2OC_6H_4$	133[g]	0	>540	<2				
37	3-$C_6H_5O(CH_2)_3OC_6H_4$	50[g]	0	>200	<5				
38	3-$C_6H_5CH_2C_6H_4$	100[g]	22	780[i]	1.3				
39	3-$C_6H_5(CH_2)_4C_6H_4$	67[g]	0	>270	<4				
40[j]	$C_6H_5CH{=}CH$	62[g]	0	>250	<5				
41	3-CH_3-5-$CH_3OC_6H_3$			530	1.9	500	0	>2000	<0.5

[a] The technical assistance of Julie Beardslee and Janet Wood is acknowledged. [b] Assayed with 1 mM choline bromide and 0.05 mM [^{14}C]acetyl-CoA as described in the Experimental Section. [c] Assayed with 1 mM [^{14}C]ACh·Cl^- as described in the Experimental Section. [d] Concn for 50% inhibition. [e] Ratio of substrate to inhibitor giving 50% inhibition. [f] Eastman Kodak Co. [g] Maximum soly. [h] Aldrich Chemical Co. [i] Estimated from inhibition at maximum soly. [j] See B. R. Baker and M. H. Doll, paper 183 of this series.

ments are further supported by the decrease in inhibition when the benzene ring is replaced by the more polar, electron-deficient pyridine ring (**19–21**).

The requirement for binding to AChE is just the opposite, that is, binding is enhanced by polar, electron-withdrawing substituents such as NO_2, CN, or aza (of the pyridyl), but not be electron-releasing groups whether polar (NH_2) or nonpolar (CH_3, Cl).

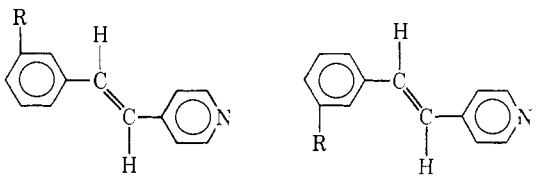
The effect of two substituents on the benzene moiety of 4-stilbazole was then studied. Introduction of 4′-Cl (**22**) on 2′-chloro-4-stilbazole (**17**) led to a loss in binding, probably because of poor steric fit. In contrast, introduction of 4′-Cl (**23**) on 3′-chloro-4-stilbazole (**11**) gave a 7-fold increase in binding; **23** was the best inhibitor in Table I, binding to the enzyme 910-fold better than choline; in fact, **23** is the most potent nonquaternary inhibitor yet known for choline acetyltransferase.

4-(β-Naphthylvinyl)pyridine (**24**) might be expected to be as good an inhibitor as 3′,4′-dichlorostilbazole (**23**), but it is actually far less effective by a factor of 45; the reason for this discrepancy became apparent when bulk tolerance studies at the 3′ and 4′ positions were made, as described later, since even an Et group (**30**) at the 4′ position leads to a 6-fold loss in binding compared to CH_3 (**6**). Introduction of a 3′,4′-CH_2O_2 moiety (**25**) led to a nearly 3-fold loss in binding compared to the parent 4-stilbazole (**1**), and a 900-fold

loss compared to 3′-methoxy-4-stilbazole (**13**); this loss is attributed primarily to the repulsion of an ether O at the 4′ position with additional secondary steric effects on fit.

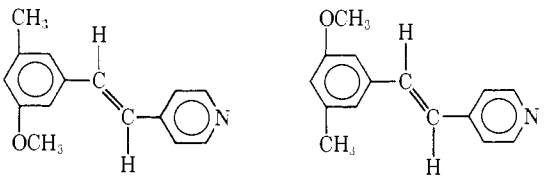
An increase in size from 4′-CH_3 (**6**) to 4′-C_2H_5 (**30**) led to a 6-fold loss in binding. Similarly increase in size from 3′-CH_3O (**13**) to 3′-C_2H_5O (**34**) led to an 18-fold loss in binding. Such results indicate a decreased bulk tolerance for the larger groups within the enzyme–inhibitor complex. However, it is sometimes possible to force an energetically unfavorable conformational change in the enzyme if by such a change net binding is increased.[19] Therefore larger groups at the 3′ and 4′ positions (**28–40**) were synthesized for enzymic evaluation; unfortunately binding was not increased by any of these large hydrophobic groups.

The question arose earlier whether increased binding by 3′-Cl (**11**) and 3′-CH_3O (**13**) was due to a point donor–acceptor interaction as previously seen with a *p*-CH_3O group on binding of 9-phenylguanine to guanine deaminase.[20] If such were the case then it is possible that Cl and CH_3O would bind in one conformation and CH_3 in a second conformation as shown by structures **42A** and **42B**; therefore **41** was synthesized for evaluation as an inhibitor of choline acetyltransferase which might bind in either conformation **41A** or **41B**.



42A

42B



41A

41B

One of 3 results could be anticipated, any one of which has a strong bearing on the enzymic environment of the Ph moiety. If there were separate binding loci for Me and MeO (**41A** or **41B**), then **41** should be a better inhibitor than either 3′-methyl-4-stilbazole (**12**) or 3′-methoxy-4-stilbazole (**13**). If the 3′-CH_3, CH_3O, and Cl were all bound to the same locus then the 5′ substituent could be in a noncontact area of the enzyme if the Ph group interacted flatly with the enzyme or was in a slot on the enzyme with one face of the benzene ring pointing away from the enzyme surface;[10] in such a case, **41** would be equal to **12** or **13** as an inhibitor. Or the Ph moiety might fit into a pocket on the enzyme; if there were bulk tolerance on both sides of the Ph ring, but binding by a group on only one side of the benzene ring, then the compound (**41**) would be equal to **12** or **13** as an inhibitor. However, if the benzene ring would fit into a pocket where there was bulk tolerance for a small group on only one side of the benzene ring, then **41** would be a much poorer inhibitor than **12** or **13**. That the latter tight fit in an enzymic pocket was the case was shown by the 40-fold loss in binding by **41** compared to **13**.

Thus the Ph moiety of 4-stilbazole binds in a flat pocket where there is room for small 3′ or 4′ substituents such as Cl or CH_3, but there is not room for both 3′ and 5′ substituents. Whether or not there is room for large substituents at the 2′ (6′) position that would reside outside the pocket remains to be determined in future work. However, it is clear that the probability of finding appreciably better binding at the 3′,4′ and 5′ positions than the 3′,4′-dichlorophenyl substituent of **23** is negligible.

The most potent compound in Table I was 3′,4′-dichloro-4-stilbazole (**23**) with I_{50} = **1.1** μM; **23** was complexed to choline acetyltransferase 910-fold better than choline. At its maximum solubility of 30 μM, **23** showed no inhibition of AChE; since 20% inhibition is readily detectable, the $I_{50} > 120$ μM. Thus the selectivity of inhibition by **23** between the two enzymes is >110.

In their elegant studies on the binding of 4-stilbazole (**1**) to choline acetyltransferase,[8–10] Cavallito, *et al.*, made the following strong points.

(**a**) The vinyl group was necessary to transmit electron-donor properties from the benzene ring to a nonquaternized pyridine ring; this transmission could also be accomplished by an acetylenic bridge, but an Et bridge destroyed the activity.

(**b**) A trans-vinyl bridge was essential since the cis-vinyl bridge caused loss of activity.

(**c**) 2-Stilbazole was also active indicating that the pyridine N did not interact in a point donor–acceptor complex, but the basic ring system interacted with some electron-donor locus on the enzyme.

(**d**) Quaternization of **1** led to a 40-fold enhanced activity, but also increased activity towards AChE.

The activity of 2-stilbazole (**43**, Table II) was confirmed, but it was only about 0.25 as active as 4-stilbazole (**1**); 3-stilbazole (**44**) was even less effective. Since the Ph moiety of 4-stilbazole appears to be complexed to the enzyme by hydrophobic bonding and since the pyridine ring can be rotated in its binding locus, it follows that the pyridine moiety will rotate to allow the Ph to have maximum hydrophobic interaction. Such rotation of a basic ring to allow maximum hydrophobic bonding by a hydrophobic side chain has been previously observed with inhibitors of dihydrofolate reductase.[21]

Although Cavallito, *et al.*,[8] showed that activity was decreased when the vinyl group of **1** or **2** was reduced to Et, the extent of this decrease could not be ascertained due to lack of solubility of the reduction products; however, it was clear at least a 33-fold loss occurred on reduction of **2**. Therefore, 3′-methyl-4-stilbazole (**12**) was reduced to **45** (Table II) and evaluated. Since **12** had I_{50} = 12 μM and **45** had I_{50} = 2000, the apparent loss in binding was 170-fold. However, this same inhibition by **45** would be observed if **45** contains 1 part in 170 of **12**, an undetectable amount; therefore, the loss in binding is equal to or greater than 170-fold when the vinyl group of 12 is reduced to Et (**45**).

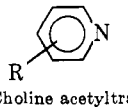
When the substituent effects on 4-stilbazole (**1**) (Table III) were compared with the substituent effects

(20) B. R. Baker and W. F. Wood, *J. Med. Chem.*, **11**, 644 (1968).

(21) B. R. Baker and H. S. Shapiro, *J. Pharm. Sci.*, **55**, 308 (1966).

TABLE II

INHIBITION[a] OF CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINESTERASE FROM RABBIT BRAIN BY



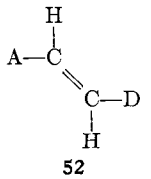
		Choline acetyltransferase[b]				Acetylcholinesterase[c]			
No.	R	Inhib. μM	% inhibn	I_{50},[d] μM	$[S/I]_{0.5}$[e]	Inhib. μM	% inhibn	I_{50},[d] μM	$[S/I]_{0.5}$
1[f]	4-C_6H_5CH=CH			470	2.1	125[g]	0	>500	<2
43[h]	2-C_6H_5CH=CH	1000	35	1800[i]	0.65	250[g]	15	>1000	<1
44[i]	3-C_6H_5CH=CH	1000	0	>4000	<0.25	500[g]	0	>2000	<0.5
45	4-(*m*-$CH_3C_6H_4CH_2CH_2$)			2000	0.50				
46	4-(*m*-$CH_3C_6H_4CH_2CH_2$) methiodide			430	2.3				

[a–i] See Table I. [i] Prepared by method A in 23% yield, mp 80–82°; mp 80–81° has been recorded by L. Horner, H. Hoffmann, H. G. Wippel, and G. Klahre, *Chem. Ber.*, **92**, 2499 (1959).

on 4-stilbazole methiodide (**47**) observed by Cavallito, *et al.*,[10] the differences were startling. Note that Cl or CH_3 substituents (**48–51**) on the methiodide (**47**) gave less than a 7-fold enhancement in binding whereas the substituent effects on 4-stilbazole (**1**) were as large as 430 with the 3,4-Cl_2 substituents (**23**).

The substituent effects on 4-stilbazole methiodide (**47**) of less than 10-fold could readily be accounted for by simple hydrophobic bonding with no electronic effects on binding; in contrast, substituent effects on 4-stilbazole must involve both hydrophobic bonding and strong electronic effects. One possibility was that the substituent(s) increased the basicity of the pyridine ring, thus enhancing its binding, since the quaternary salts are already such strong bases that substituents could not possibly make them stronger. This possibility did not seem probable since halogen substitution makes aniline a weaker base, whereas Me substitution makes aniline a slightly stronger base. Nevertheless, the pK_a's of the substituted 4-stilbazoles were determined (Table III). The substituents had little effect on basicity of the pyridine N, showing electron transmission through the vinyl bridge did not occur. This lack of correlation of pK_a and binding indicates that the effect of substitution on the benzene ring is much more likely on binding of the styryl moiety and not the pyridine moiety of 4-stilbazole.

Cavallito, *et al.*,[8–12] have emphasized throughout their work that the most important features of the binding of *trans*-4-stilbazole to choline acetyltransferase were the electron-donor properties of the benzene ring, the electron-acceptor properties of the pyridine ring, and the easy transmission of electrons through a transvinyl or an acetylenic group; this they pictured as in **52** where A was an electron-poor pyridine or quinoline ring and D was an electron-rich benzene or naphthalene ring. They stated that the main binding could be



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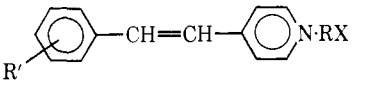
charge–transfer complexing of A and D to the enzyme, augmented by hydrophobic bonding of the nonpolar donor (D) ring. Such an argument has the drawback that charge–transfer complexing to an enzyme will have less than **1** kcal/mole of energy for each interaction,[18] whereas hydrophobic bonding by a benzene

TABLE III

COMPARISON OF SUBSTITUENT EFFECTS ON INHIBITION OF CHOLINE ACETYLTRANSFERASE BY 4-STILBAZOLE AND ITS METHIODIDE



No.	R′	RX	I_{50}, μM	Increment by R′	pK_a
1[a]	H	Base	470		5.6[e,f]
1[b]	H	Base	600		5.7[b]
47[c]	H	CH_3I	15		
5[a]	4-Cl	Base	170	2.8	5.8[e]
48[c]	4-Cl	CH_3I	7	2.1	
6[a]	4-Me	Base	55	8.6	5.5[e]
49[c]	4-Me	CH_3I	20	0.75	
11[a]	3-Cl	Base	7.8	60	5.6[e]
50[c]	3-Cl	CH_3I	2.3	6.5	
23[a]	3,4-Cl_2	Base	1.1	430	5.5[e]
51[c]	3,4-Cl_2	CH_3I	3.7	4.1	
2[d]	2,3-Benzo	Base	25	24	5.5[g]
3[c]	2,3-Benzo	CH_3I	0.47	32	
12[a]	3-Me	Base	12	39	
13[a]	3-OCH_3	Base	13	36	5.8[e]
24[a]	3,4-Benzo	Base	52	9.0	5.8[e]

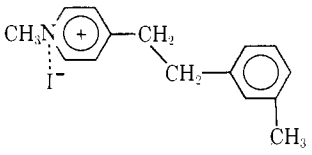
[a] Data from Table I. [b] Data from J. C. Smith, C. J. Cavallito, and F. F. Foldes, *Biochem. Pharmacol.*, **16**, 2438 (1967). [c] Data from C. J. Cavallito, H. S. Yun, T. Kaplan, J. C. Smith, and F. F. Foldes, *J. Med. Chem.*, **13**, 221 (1970). [d] Data from C. J. Cavallito, H. S. Yun, J. C. Smith, and F. F. Foldes, *ibid.*, **12**, 134 (1969). [e] Determined by pH of half neutralized 0.1 mM soln in 10% DMSO. [f] 2- and 3-stilbazole had pK_a 5.9 and 5.1, respectively; pyridine has pK_a = 5.2. [g] Data from H. L. White and C. J. Cavallito, *Biochim. Biophys. Acta*, **206**, 242 (1970).

ring could have as much as 6 kcal/mole of binding energy;[18] therefore the charge–transfer characteristics of the two rings **52** could only account for a minor contribution to binding to the enzyme.

At this point another paper by Cavallito, *et al.*,[22] appeared where Hückel MO and Hansch π–σ calculations[17] were made to try to gain further insight into the mode of binding of the quaternarized styryl pyridines to choline acetyltransferase. Their conclusions were then modified. It was proposed that these compounds were complexed to the enzyme by hydrophobic and electron-donor contributions of the aryl moiety and electron-acceptor interaction of the pyridinium moiety; no specific binding contribution could be ascribed to the vinyl bridge other than transmission of electrons between the two rings and facilitation of co-

(22) R. C. Allen, G. L. Carlson, and C. J. Cavallito, *J. Med. Chem.*, **13**, 909 (1970).

planarity of the inhibitor. We have already shown that the transmission of electrons from one ring to the other is of little consequence (see discussion of Table III). Although Cavallito, *et al.*,[8,9] did show that the ethylenic bridge was essential for binding by styrylpyridines, they did not show that the ethylenic bridge was essential for binding of the quaternized derivatives. Therefore, **46** was prepared for assay (Table II). A large loss in binding occurred compared to **12** and **50** (Table III). It seems unlikely that the difference in



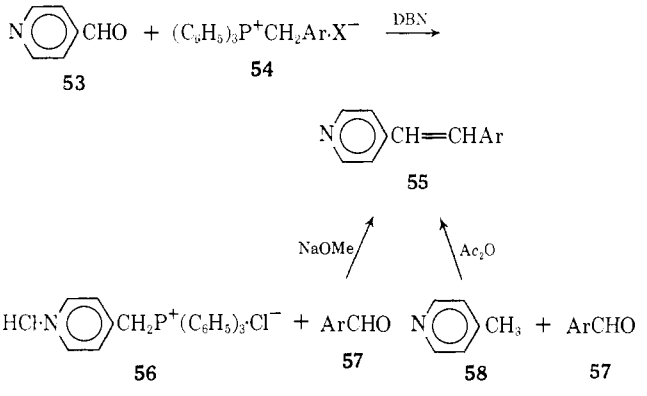
46

ground-state conformation between 4-stilbazole and 4-phenethylpyridine could account for this large loss in binding; probably less than 1 kcal/mole of energy would be required to move the two rings of **46** from parallel planes to the coplanarity of a 4-stilbazole such as **52**. Therefore the vinyl bridge is most probably involved in direct binding to the enzyme.

A number of possibilities for direct binding of the vinyl bridge are feasible such as direct π bonding or by partial polarization of vinyl group with a partial minus charge on the pyridine side and partial plus charge on the benzene side of the vinyl bridge. Further studies are underway to distinguish between the possible modes of binding of the styryl moiety of 4-stilbazole to choline acetyltransferase.

Of considerable practical importance is the emergence from this current study of several potent inhibitors of choline acetyltransferase that are nonquaternized and should therefore be able to pass the blood–brain barrier and other membranes; these are the 3′,4′-Cl_2 (**23**), 3′-Cl (**11**), 3′-OCH_3 (**13**), and 3′-CH_3 (**12**) derivatives of 4-stilbazole which bind to the enzyme 910-, 130-, 77-, and 83-fold better than the substrate, choline (Table I). Furthermore these compounds are poor inhibitors of AChE, thus showing good selectivity between these two enzymes in the nerve ending.

Chemistry.—Three general methods for synthesis of the 4-stilbazole derivatives (**55**) in Table IV were employed. Two of these methods involved the Wittig



reagents **54** or **56**;[23] the choice of method depended primarily upon the relative availability of the arylaldehyde (**57**) or benzyl halide precursor to **54**. The Wittig reaction usually gave a mixture of *cis*- and *trans*-stilbazoles (**55**) which were separated. The *trans*-stilbazole of a pair was identified by its uv absorption; the ratio of the height of the long wavelength peak (~310 mμ) to the height of the short wavelength (~220 mμ) is greater than one in the trans isomer and less than one in the cis isomer.[24]

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Each analytical sample had an ir spectrum compatible with its structure and moved as one spot on tlc on Brinkmann silica gel GF with EtOAc. All anal. samples gave combustion values for C, H, or C, H, and N within 0.4% of theoretical.

3-Chlorobenzyltriphenylphosphonium Chloride (60).—A mixt of 11.97 g (74.8 mmoles) of α,*m*-dichlorotoluene, 19.6 g (74.8 mmoles) of triphenylphosphine, and 100 ml of xylene were refluxed for 15 hr. The mixt was cooled and the white crystals collected. Two recrystns from EtOH–petr ether (bp 65–110°) gave the anal. sample; yield, 23.0 g (72%), mp 326–328°. *Anal.* ($C_{25}H_{21}Cl_2P$) C, H.

3′-Chloro-4-stilbazole (11) Hydrochloride. Method A.—A mixt of 3.0 g (27 mmoles) of 4-pyridinecarboxaldehyde, 11.4 g (27 mmoles) of 3-chlorobenzyltriphenylphosphonium chloride, and 1.67 g (30 mmoles) of NaOMe in 100 ml of MeOH was stirred at room temp for 20 hr. The mixt was poured into 100 ml of 4 *N* HCl. The acidic soln was washed with three 50-ml portions of C_6H_6 and basified with 50% NaOH and the product extd with three 50-ml portions of C_6H_6. The C_6H_6 layer was washed with three 50-ml portions of H_2O and dried (Na_2SO_4) and the solvent was removed *in vacuo*. The crude oil was dissolved in 100 ml of Et_2O and the product was pptd with dry HCl gas. The HCl salt was collected by filtration and recrystd from EtOH–petr ether (bp 65–110°). For additional data see Table IV.

Crystalline free bases were recrystd from the appropriate solvent (see Table IV). Oils were isolated as either the HCl salt from Et_2O or the TsOH salt from Et_2O.

2′-Methoxy-4-stilbazole (18) Picrate. Method B.—To a stirred soln of 3.15 g (7.4 mmoles) of 4-picolyltriphenylphosphonium chloride hydrochloride[23] and 1.0 g (7.4 mmoles) of *o*-anisaldehyde in 50 ml of DMF was added 1.86 g (14.8 mmoles) of 1,5-diazabicyclo[4.3.0]nonene (DBN). After 15 hr the mixt was poured into 50 ml of H_2O. After acidification with 4 *N* HCl, the extn procedure described in method A was followed. The benzene was removed *in vacuo*. The residue was then taken up in 25 ml of EtOH and added to 0.5 g (2.1 mmoles) of picric acid in 25 ml of EtOH. The salt was collected and two recrystns from EtOH–H_2O gave the anal. sample. See Table IV for additional data.

4′-Acetamido-4-stilbazole (9). Method C.—A mixt of 10 g (61 mmoles) of 4-acetamidobenzaldehyde, 5.67 g (61 mmoles) of 4-picoline, and 30 ml of Ac_2O was refluxed overnight. The cooled mixt was poured into 100 ml of 2 *N* HCl and extd with two 50-ml portions of C_6H_6. The HCl layer was cooled and then basified with 50% NaOH. The crude product was collected by filtration and recrystd twice from EtOH–H_2O. See Table IV for additional data. HCl and TsOH salts were prepared as described in method A.

4′-Amino-4-stilbazole (8).—A mixt of 4.0 g (16.8 mmoles) of **9**, 300 ml of EtOH, 30 ml of concd HCl, and 50 ml of H_2O was heated on a steam bath for 2 hr, cooled, and basified with 50% NaOH. The yellow ppt was collected by filtration and recrystd from EtOH. See Table IV for additional data.

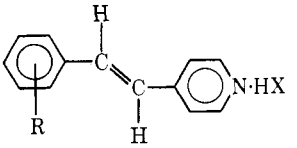
3′-Amino-4-stilbazole (14).—A mixt of 2.0 g (8.8 mmoles) of 3′-nitro-4-stilbazole (**16**) and 50 mg of 10% Pd/C in 200 ml of abs EtOH was shaken under 2–3 atm H_2 until the calcd quantity of H_2 was taken up. The filtered soln was evapd *in vacuo*. Tlc of the crude reduction product with *n*-BuOH–HOAc–H_2O (4:1:5) showed 3 spots. The anal. sample was obtained by 2 recrystns from EtOH–H_2O. The uv spectrum in MeOH showed a peak at 310 mμ characteristic of a 4-stilbazole. See Table IV for additional data.

(23) B. R. Baker and M. H. Doll, submitted for publication, paper 183 of this series.

(24) G. Cauzzo, G. Galiazzo, M. Mazzucato, and N. Mongiat, *Tetrahedron*, **22** (2), 689 (1966).

TABLE IV

PHYSICAL PROPERTIES OF



No.		HX	Method	% yield[a]	Mp, °C	Formula[b]
5	4-Cl	TsOH	A[c]	11	267–268[d]	
6	4-CH_3	Base	A[c]	64	150–151[e]	
7	4-OCH_3	Base	C	17[f]	134–136[g]	
8	4-NH_2	Base	Exp	72[h]	279–281 dec	$C_{13}H_{12}N_2$
9	4-NHAc	Base	C[i]	31	218–219	$C_{15}H_{14}N_2O$
10	4-NO_2	Base	C	37	171–172[j]	
11	3-Cl	HCl	A[k]	62[l]	229–230	$C_{13}H_{10}ClN \cdot HCl$
12	3-CH_3	TsOH	A[c]	18[m]	201–202	$C_{14}H_{13}N \cdot C_7H_8O_3S$
13	3-OCH_3	Base	C	21[n]	61–63	$C_{14}H_{23}NO$
14	3-NH_2	Base	Exp	4[i]	191–192	$C_{13}H_{12}N_2$
15	3-CN	Base	A[o]	2[i]	148–149	$C_{14}H_{10}N_2$
16	3-NO_2	TsOH	A[c]	21[i]	151–152	$C_{13}H_{11}N_2O_2 \cdot C_7H_8O_3S$
17	2-Cl	HCl	A[p]	4.5[q]	227–230 dec	$C_{13}H_{10}ClN \cdot HCl$
18	2-OCH_3	Picrate	B	17[i]	168–170	$C_{14}H_{13}NO \cdot C_6H_3N_3O_7$
22	2,4-Cl_2	Base	A[r]	10[s]	70–71	$C_{13}H_9Cl_2N$
23	3,4-Cl_2	TsOH	A	6.3[l]	267–268	$C_{13}H_9Cl_2N \cdot C_7H_8O_3S$
24	3,4-Benzo	Base	C	4.7[i,t]	162–163	$C_{17}H_{13}N$
25	3,4-Methylenedioxy	Base	C	35[u]	95–100	$C_{14}H_{11}NO_2$
26	3,4,5-$(OCH_3)_3$	Picrate	B	4.7[i]	247–248	$C_{16}H_{17}NO_3 \cdot C_6H_3N_3O_7$
27	4-OCH_2CH_3	Base	C	30[s]	150–151	$C_{15}H_{15}NO$
28	4-$OCH_2C_6H_5$	Base	C	57[s]	184–185	$C_{20}H_{17}NO$
29	4-$O(CH_2)_3C_6H_5$	TsOH	C[v]	7.8[h]	194–195	$C_{22}H_{21}NO \cdot C_7H_8O_3S$
30	4-C_2H_5	Base	C[w]	23[s]	96–97	$C_{15}H_{15}N$
31	4-$CH_2C_6H_5$	Base	C[x]	15[s]	105–106	$C_{20}H_{17}N$
32	4-CH=CH—C_6H_5	HCl	C[y]	8.3[i]	265–266	$C_{21}H_{17}N \cdot HCl$
33	4-$(CH_2)_4C_6H_5$	Base	C[x]	31[u]	127–130	$C_{23}H_{23}N$
34	3-OC_2H_5	TsOH	C[v]	7.4[n]	158–159	$C_{15}H_{15}NO \cdot C_7H_8O_3S$
35	3-OC_5H_{11}-*i*	HCl	C[v]	40[h]	193–194	$C_{18}H_{21}NO \cdot HCl$
36	3-$OCH_2C_6H_5$	Base	C	31[s]	78–81	$C_{20}H_{17}NO$
37	3-$O(CH_2)_3OC_6H_5$	HCl	C[v]	50[l]	169–171	$C_{22}H_{21}NO_2 \cdot HCl$
38	3-$CH_2C_6H_5$	Base	C[x]	7.8[s]	91–92	$C_{20}H_{17}N$
39	3-$(CH_2)_4C_6H_5$	Base[x,z]	B[aa]	14	51–54	$C_{23}H_{23}N$
41	3-OCH_3-5-CH_3	HCl	A[k]	41[bb]	235–238	$C_{15}H_{15}NO \cdot HCl$

[a] Yield is of anal. pure material and is minimum. [b] Anal. for C, H, N within 0.4% of theory where empirical formulas are given. [c] For starting Wittig reagent (**54**) see C. E. Griffin and M. Gordon, *J. Organomet. Chem.*, **3** (5), 414 (1965). [d] Free base had mp 111–112°; lit. [A. R. Katritzky, D. J. Short, and A. S. Boulton, *J. Chem. Soc.*, 1516 (1960)] mp 113°. [e] Lit.[d] mp 150–151°. [f] TsOH salt was purified, then converted into free base. [g] Lit.[d] mp 135.5–136.5°. [h] Recrystd from EtOH. [i] Recrystd from EtOH–H_2O. [j] Lit.[d] mp 171–172°. [k] See Experimental Section for Wittig reagent. [l] Recrystd from EtOH–petroleum ether (bp 65–110°). [m] Recrystd from H_2O. [n] Recrystd from EtOAc. [o] For starting Wittig reagent (**54**) see B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **12**, 408 (1969). [p] For starting Wittig reagent (**54**) see B. R. Baker, B. T. Ho, and G. J. Lourens, *J. Pharm. Sci.*, **56**, 737 (1967). [q] Recrystd from EtOAc–EtOH. [r] For starting Wittig reagent (**54**) see W. P. Keaveney and D. J. Hennessey, *J. Org. Chem.*, **27**, 1057 (1962). [s] Recrystd from petr ether (bp 65–110°). [t] Recrystd from hexane after chromatography on silica gel with PhH–hexane (1:4). [u] Recrystd from EtOAc–petroleum ether (bp 65–110°). [v] Starting aldehyde (**57**) made by alkylation of the appropriate hydroxybenzaldehyde in DMF–K_2CO_5 by the general method of B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **11**, 245 (1968). [w] Starting aldehyde prepared from *p*-EtC_6H_4MgBr and $(EtO)_3CH$ by the general method of M. H. Klouwen and H. Boelens, *Recl. Trav. Chim. Pays-Bas*, **79**, 1022 (1960). [x] Starting aldehyde made by method D. [y] See Experimental Section for starting aldehyde. [z] Aldehyde prepd from *m*-$C_6H_5(CH_2)_4C_6H_4CN$ by Red-Al; see B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **12**, 408 (1969). [aa] Isolated as picrate; treatment with Dowex (Cl^-) in EtOH, gave the base on evapn. [bb] Recrystd from EtOH–C_6H_6.

4-(4-Phenylbutyl)benzaldehyde. Method D.—To a cooled (5°) soln of 7.2 g (30 mmoles) of 1-(4-cyanophenyl)-4-phenylbutane[25] in 150 ml of PhH was added dropwise a soln of $Na[Al(OCH_2CH_2OCH_3)_3H_2]$ (Red-Al) prepd by diln of 8.83 g of a 70% soln in C_6H_6 with 40 ml of C_6H_6. The temp was maintained below 10° during the addition. After 15 min, 50 ml of 4 *N* HCl was added dropwise. The org layer was sepd and the acidic layer extd with two 50-ml portions of C_6H_6. The combined C_6H_6 exts were washed free of acid and dried (Na_2SO_4) and the solvent was removed *in vacuo*. Tlc of the crude oil showed one major and two minor spots. One of the minor spots was identified as starting material. Ir and nmr were consistent with the structure of 4-(4-phenylbutyl)benzaldehyde. The crude product was used without further purification.

(25) B. R. Baker and E. H. Erickson, *J. Med. Chem.*, **12**, 408 (1969).

3-Methoxy-5-methylbenzytriphenylphosphonium Bromide (59).—A mixt of 10 g (73.5 mmoles) of 3,5-dimethylanisole, 13 g (73.5 mmoles) of NBS, 500 mg of benzoyl peroxide, and 150 ml of CCl_4 was refluxed for 1 hr. The mixt was cooled and filtered. The filtrate showed a single spot on tlc which gave a positive test for active halogen. The CCl_4 was removed *in vacuo*. To the residue was added 150 ml of xylene and 19 g (72.5 mmoles) of Ph_3P, then the soln was refluxed for 90 min. The product was collected by filtration from the hot reaction mixt; two recrystns from EtOH–H_2O gave white crystals suitable for further transformation, yield 20.5 g (59%). An additional recrystn from EtOH–H_2O gave the anal. sample, mp 277–277.5° dec. *Anal.* ($C_{27}H_{26}BrOP$) C, H.

4-Stilbenecarboxaldehyde.—To a mixt of 3.22 g (23.7 mmoles) of 1,4-phthalaldehyde and 0.43 g (8 mmoles) of NaOMe in 25 ml of MeOH was added dropwise a soln of 3.1 g (7.9 mmoles) of benzyl-

triphenylphosphonium chloride in 15 ml of MeOH. After 2 hr of stirring at ambient temp, the mixt was poured into 300 ml of H_2O. H_2O was decanted from the yellow solid which was digested in 100 ml of hot H_2O. After 2 digests, the yellow solid was collected by filtration and recrystd from EtOH–H_2O to give 1.75 g (91%) of light yellow needles, mp 115–116°; lit.[26] mp 116°, prepd by an alternate route.

4-(3-Methylphenethyl)pyridine (45) *p*-Toluenesulfonate.—A mixt of 2.0 g (10.3 mmoles) of **12** as the free base and 50 mg of 10% Pd/C was reduced as described for **14**. After filtration of the catalyst and evapn of the solvent, 2.0 g (10.4 mmoles) of TsOH in 100 ml of Et_2O was added to the residue. The salt was collected and recrystd twice from EtOAc; yield, 3.46 g (92%), mp 112–113°. *Anal.* ($C_{21}H_{23}NO_3S$) C, H, N.

4-(3-Methylphenethyl)pyridinium Methiodide (46).—A mixt of 6.14 g (31 mmoles) of **45** and 10 ml of MeI was heated on a steam bath for 30 sec when it solidified. The solid was heated 5 min more, than recrystd from Me_2CO–petr ether (bp 65–110°)–MeOH; yield, 8.37 g (81%), mp 166–167°. *Anal.* ($C_{15}H_{18}IN$) C, H, N.

Preparation of Enzyme and Assay Methods.—The enzyme prepn was a modification of the method of Potter, *et al.*,[14] used for rat brain. A mixt of 2.0 g of rabbit brain Me_2CO powder and 40 ml of ice-cold 0.1 m*M* Versene was homogenized in a pre-cooled head of a Waring blender for 2 min. After the addition of 50 μl of 1 *M* NH_4OH and 0.45 ml of *n*-BuOH, the mixt was blended an additional 30 sec. It was centrifuged at 20,000 rpm for 20 min in a No. 40 rotor of a Spinco L centrifuge. The supernatant was sepd and adjusted to pH 5 with 1 *M* HOAc (about 0.2 ml). The mixt was centrifuged at 20,000 rpm for 15 min and the supernatant rejected. The pellet was carefully rinsed with 5 ml of ice-cold H_2O, then stirred for 20 min with 0.2 *M* KCl at 0° for 15 min. The mixt was centrifuged at 20,000 rpm for 20 min. The supernatant (18 ml) was stored at 3°. Choline acetyltransferase activity gradually decreased, but was sufficiently active up to storage for 1 month. The AChE activity was stable over several months.

Choline acetyltransferase activity was measured by modification of the method of McCaman and Hunt;[15] the assay was run in the presence of 10% DMSO, and inhibitors were added in this solvent. The assay mixt contained 1 m*M* choline, 0.1 m*M* eserine, 0.1 *M* KCl, 0.025 *M* Tris buffer, and 0.1 m*M* acetyl CoA (5 mCi/mmole). The final Reinecke salt in Me_2CO was spotted on a glass filter paper, dried, and counted in PhMe containing 0.4% PPO and 0.01% dimethyl POPOP.

AChE activity was measured by modification of the method of Potter;[16] the assay was run in the presence of 2.5% MeOEtOH and inhibitors were dissolved in 25% MeOEtOH since 10% DMSO completely inhibited the reaction. The assay contained 1 m*M* $ACh \cdot Cl^-$ (0.9 mCi/mmole), 0.05 *M* Tris buffer, and 0.02 *M* $MgCl_2$.

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Inhibition of Phenethanolamine *N*-Methyl Transferase by Ring-Substituted α-Methylphenethylamines (Amphetamines)

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Phenethanolamine *N*-methyl transferase (PNMT) transfers a Me group from *S*-adenosylmethionine to phenethylamines with an OH group β to the N. Phenethylamines (including α-methylphenethylamines) without such a β substitution combine with and inhibit the enzyme. Amphetamines with various aromatic substituents were studied as inhibitors; there was greater than a 1000-fold range in their inhibitor potency. The inhibitor activity of these compounds showed a correlation with the Hammett σ and π (a lipophilic parameter derived from the partition coefficient) associated with the aromatic substituent. The *d* isomers were more active as inhibitors than *l* isomers of amphetamines. *d*-3,4-Dichloroamphetamine was the most active inhibitor in the series and is the most potent inhibitor of PNMT reported to date; its inhibition was reversible and competitive. Inhibitors of PNMT that are effective *in vivo* should be of pharmacological importance.

Phenethanolamine *N*-methyl transferase (PNMT) transfers a Me group from *S*-adenosylmethionine to a Me acceptor, which apparently has to be either a phenethanolamine or a phenylethylenediamine.[1-3] The physiological role of PNMT is to convert norepinephrine into epinephrine, primarily in the adrenal medulla.[4] Although the physiological effects of norepinephrine and epinephrine are qualitatively similar in general, there are numerous differences in the responses of various target organs to these two catecholamines.[5-7] Thus an inhibitor of PNMT which altered the ratio of epinephrine:norepinephrine in the adrenal gland ought to be an interesting pharmacological tool and at least potentially useful as a drug. However, few studies on PNMT inhibitors have been published. PNMT is inhibited by sulfhydryl binding agents.[1,8,9] Fuller and Hunt[2] reported that some phenethylamines structurally related to substrates but lacking the β-OH essential for substrate activity were inhibitory to PNMT. Krakoff and Axelrod[10] reported the inhibition of PNMT by several amines, among which the monoamine oxidase inhibitor tranylcypromine was one of the most potent inhibitors.

Both the reports by Fuller and Hunt and by Krakoff and Axelrod showed that amphetamine was a relatively weak PNMT inhibitor. We describe here the inhibition by a series of substituted amphetamines, the most active of which represent the most potent PNMT inhibitors reported up to this time.

Experimental Section

The enzyme prepn and the method of enzyme assay were as reported previously.[11] An $(NH_4)_2SO_4$ fraction of the high-speed

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