Improved Delivery through Biological Membranes. 3. Delivery of N-Methylpyridinium-2-carbaldoxime Chloride through the Blood–Brain Barrier in Its Dihydropyridine Pro-Drug Form†,1

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Administration of N-methyl-1,6-dihydropyridine-2-carbaldoxime hydrochloride, the pro-drug form of 2-PAM, resulted in an average of 13-fold increase in the amount of 2-PAM delivered into the brain of mice as compared to the administration of 2-PAM. The pro-drug which crossed the BBB resulted in a dramatic increase in the reactivation of AChE blocked by DFP. In vivo studies of the "aging" of the phosphorylated AChE in the brain of mice could also be studied using pro-2-PAM.

It has been shown[1] that applying the dihydropyridine–pyridine type redox pro-drug system on N-methylpyridinium-2-carbaldoxime chloride (2-PAM) a significant improvement in the distribution and elimination characteristics of the drug can be achieved. Thus, the N-methyl-1,6-dihydroppyridine-2-carbaldoxime hydrochloride (pro-2-PAM) synthesized,[2] when delivered iv to dogs, distributes easily and preferentially to lipoidal tissues, followed by a rapid and quantitative transformation into 2-PAM, by an oxidative process. The overall result was that 2-PAM was delivered into lipoidal tissues essentially unavailable for 2-PAM administered as such. On the other hand, an extended biological half-life of 2-PAM was also achieved, without resulting, however, in any new, unwanted metabolic product.

These facts would suggest that pro-2-PAM will also penetrate the blood–brain barrier (BBB), the most unpenetrable lipoidal barrier, thus resulting in delivery of 2-PAM into the brain and reactivation of the acetylcholinesterase (AChE) blocked by anticholinesterase poisons.

In the present paper, the delivery through the BBB of 2-PAM is described, using the above dihydropyridine–pyridine type redox pro-drug approach. Penetration into the CNS is part of the distribution characteristics of any drug.[3] In the case of 2-PAM, however, a large number of specific studies on the ability of 2-PAM to enter the brain were done, because of the apparent importance of reactivating the blocked AChE in the brain.

It is generally agreed that asphyxiation is the ultimate cause of death in mammals[4,5] after poisoning with an anticholinesterase agent, such as organophosphates. There is some uncertainty, however, about the relative importance of the four different direct or indirect mechanisms involved: (a) bronchoconstriction; (b) lowered blood pressure; (c) neuromuscular block of the respiratory muscles; and (d) failure of the respiratory center in the brain. In some cases, such as in cats poisoned with TEPP (tetraethylpyrophosphate) it was suggested[6] that factor d is the most important, while in rabbits treated with DFP (diisopropyl fluorophosphate) or TEPP, the peripheral a, b, and c seem to be of primary importance.[7] In the monkey, central failure seems to be the sole cause of cessation of ventilation, the bronchoconstriction and neuromuscular block being insignificant at the time of failure.[5] As a result of a detailed study in nine mammalian species, it was concluded[5] that "the central failure seems to be the predominant feature in most instances, but the detailed picture varies with the species studied, the drug used, and the dosage administered".

Besides the obvious importance of the level of brain cholinesterase activity in the survival of the intoxicated patients, no experiments have been conducted to study the influence of unreactivated AChE on the brain functions of the surviving patient. Very recent data indicate[8] that, in humans, psychiatric sequelae persist for many weeks after intoxication with organophosphates despite an adequate treatment with atropine and 2-PAM. The sequelae were interpreted as the direct result of excess cholinergic stimulation in the CNS, as a result of the accumulation of acetylcholine due to the blocking of the AChE.

As an ionic compound, 2-PAM would not be expected to cross the BBB and thus it does not reactivate the blocked brain AChE. Thus, there was no 2-PAM found[9] in the cerebrospinal fluid of man 70 min after it had been administered iv. However, Firemark et al.,[10] by autoradiography of the rat brain following iv injection of 20 mg/kg of 2-PAM labeled with a ^{14}C carbonyl side chain, concluded that unmetabolized 2-PAM entered some anatomical areas of the brain. Other studies[10,11] have also indicated various degrees of reactivation in the cerebral cortex of rats poisoned with Paraoxon (diethyl 4-nitrophenyl phosphate). However, in assessing reactivation of AChE in whole brain homogenates of mice and rats it was found that hardly any reactivation took place in the CNS when pyridiniumcarbaldoximes are given in amounts sufficient to produce a considerable reactivation at peripheral sites.[12-14]

A recent hypothesis[15] states that a certain minimal AChE activity level is necessary for the survival of an organism poisoned by an organophosphorus compound. This "minimal level" is different for different parts of the brain. Even if this hypothesis would hold and small quantities of 2-PAM which gain access to some parts of the CNS might be enough to elicit pharmacologic effects and save the life of the intoxicated patient, there still remain two major problems derived from the low brain AChE level: (1) the impairment of certain brain functions due to the consequences of the low AChE level, and (2) high susceptibility to further intoxication; i.e., the "minimal level" could be lowered by a small amount of poison. This susceptibility would last for a long time, since the regeneration of the blocked and subsequently "aged"[16] AChE is a very slow process.

All the above facts only emphasize the importance of delivering 2-PAM through the BBB. Theoretically, there are various possibilities how this could be realized. A recent excellent review[17] describes the factors influencing the changes in the permeability of the BBB. Changes in permeability are, for example, caused by brain tumors,[18]

† Dedicated to the memory of Professor Edward E. Smissman.

Table I. Protection of 2-PAM and Pro-2-PAM against Phospholine Iodide Toxicity in Mice

Group	2-PAM, mg/kg	Pro-2-PAM, mg/kg	Phospholine iodide, mg/kg	Normal saline, ml	Survived
I			0.5[a]	0.2	1/10
II	50		0.5		10/10
III		50	0.5		10/10
IV		50		0.2	10/10

[a] About 2 × LD_{50}.

but they can also be induced. Three possible ways for promoting entrance of drugs that normally penetrate with great difficulty into the CNS were suggested.[3] (a) One approach is to use pharmacological agents that increase the permeability of the BBB. A number of substances such as snake venoms, ethanol, etc., have been shown to damage the BBB so that a normally excluded test substance will enter. However, the barrier is cellular in nature[3] and chemical agents which damage it must have a similar action on most other body cells. (b) A more direct approach is administration of the drug directly into the CSF, which approach, however, encounters two major difficulties, uneven distribution and slow diffusion, not mentioning the technical problems related to the administration. The method is still very useful in verifying certain theories, such as the effect of delivering drugs through the BBB. Thus, cerebral intraventricular injections of 2-PAM were reported[19,20] to enhance strikingly its therapeutic efficacy against alkyl phosphate poisoning. (c) Based on some successes in penetrating other lipoidal membranes such as the intestinal epithelium, it was suggested[3] that the best approach to the problem of penetrating the BBB is to develop drugs that are lipid soluble. It was further suggested that acetylated derivatives of certain nonlipoidal drugs would penetrate the CNS more readily than do the parent compounds.

As for ionic species, such as quaternary ammonium compounds, an interesting approach has recently been suggested,[21] which has, however, a very limited practical value. The approach is based on the delivery of a tertiary haloalkylamine which can pass into the brain in the undissociated form, which will then cyclize to the quaternary derivative. The validity of the concept was proved on a model compound, i.e., *N*-(5'-chloropentyl-*N*-methylaminoaceto)-2,6-xylidide, but no direct application for specific drugs of this approach can be foreseen.

Results and Discussion

Limited biopharmaceutical studies[1] have already indicated that pro-2-PAM converts to 2-PAM in vivo quickly and quantitatively after iv administration to dogs. This means that pro-2-PAM should be at least as good a protector against anticholinesterase agents as 2-PAM itself. This was verified using white mice. It has been reported that 2-PAM is highly effective against diethoxyphosphinylthiocholine iodide (phospholine iodide) poisoning.[22] The LD_{50} of this phosphate type anticholinesterase was found to be 0.229 mg/kg after ip administration to mice.[23] In the present study, 40 mice were divided into four groups of ten each and treated as indicated in Table I. Both the poison and the drug were administered.

Both 2-PAM and pro-2-PAM were administered 10 min prior to intoxication with 2 × LD_{50} of phospholine iodide. The mice in group I were a control group for toxicity of phospholine iodide, while group IV was a control for the toxicity of pro-2-PAM.

At equivalent doses, pro-2-PAM and 2-PAM apparently afforded the same protection.

Table II. Total Radioactivities in Mice Brains Calculated as 2-PAM and Pro-2-PAM, 15 min after Iv Administration of 5.0 mg/kg of the Corresponding Radiolabeled Materials

2-PAM iodide			Pro-2-PAM		
Brain wt (wet), mg	Total amt, μg	Concn, mg %	Brain wt (wet), mg	Total amt, μg	Concn, mg %
469.7	1.23	0.262	407.9	45.15	11.070
453.7	0.97	0.214	472.7	11.92	2.522
444.0	1.12	0.252	479.5	46.64	9.727
439.6	1.44	0.328	427.1	6.74	1.578
442.8	1.64	0.370	451.2	24.60	5.452
449.8	2.11	0.469	428.6	12.08	2.818
437.6	3.90	0.891	465.0	9.32	2.011
	Mean	0.398 mg %		Mean	5.025 mg %
	SE	±0.098 mg %		SE	±1.60 mg %

It was reported that 3-PAM (in which the aldoxime group has a pK_a of 9.2[24]), as well as the tetrahydro derivatives of 3- and 4-PAM (in which the pK_a of the oximes are 10.84 and 10.91,[25] respectively), lacks the ability to protect against anticholinesterase agents.[25,26] Likewise, pro-2-PAM with an aldoxime pK_a above 10 is also expected to have little ability to reactivate phosphorylated AChE in vitro. Thus, the observed protection by pro-2-PAM must be attributed to 2-PAM which is formed in vivo from pro-2-PAM.

Next, the ability of pro-2-PAM to penetrate the BBB was studied using radiolabeled 2-PAM and pro-2-PAM, respectively, administering the drugs iv to white mice. One group of seven mice was injected iv with a dose of 5.0 mg/kg of [^{14}C]-2-PAM iodide, while another group was given a similar dose of radiolabeled pro-2-PAM. Fifteen minutes later the animals were sacrificed and the amount of radioactivity in the whole brain was determined. The results are listed in Table II.

Calculating from the total radioactivity found in mice brains after treating them with ^{14}C-radiotagged 2-PAM and pro-2-PAM, it was found that an average of 13 times more pro-2-PAM than 2-PAM had penetrated into the brain. In order to test whether the observed radioactivity of the mice treated with the pro-drug was attributed to pro-2-PAM or 2-PAM or to some other metabolites, radiochromatography of the corresponding brain homogenates was performed. The major component found was identified as being 2-PAM, as indicated in Figure 1, having an R_f of 0.53[27] in the eluent used. It is possible that at the time when the animals were sacrificed, part of the radioactivity was due to the pro-drug which was oxidized to 2-PAM during the process of preparation for chromatography. This possibility, however, does not change the final conclusion. It is obvious that the radioactivity was not due to metabolites which could not be converted to 2-PAM. The average amount of pro-2-PAM (from the total radioactivity) found in the brains was 22.48 μg. The average dose per mouse was 1.5 mg, thus about 1.50% of the dose was delivered to the brain, while the brain weight (wet) was 1.49% of the total mice body.

From these results, it can be assumed that the oxidation of the pro-drug in mouse blood was not too fast to prevent its penetration into the brain. This supports the previous assumption[1] that the very fast "input" of 2-PAM when pro-2-PAM was given is a complicated process which includes steps of fast oxidation and distribution.

Does the increased delivery of 2-PAM by pro-2-PAM into the brain result in an increased ability of 2-PAM to reactivate phosphorylated AChE in the brain? To answer

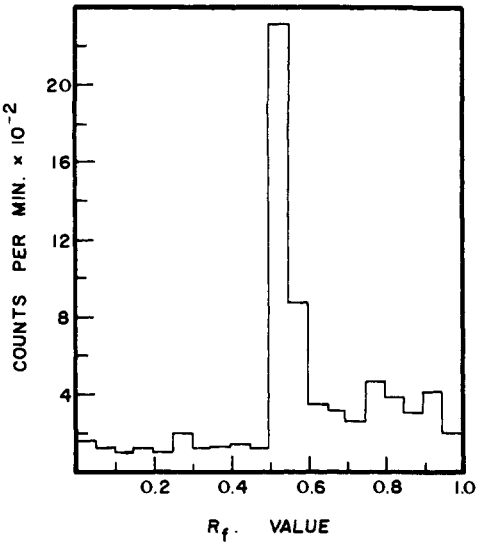

Figure 1. Radiochromatography of brain extract obtained from [^{14}C]pro-2-PAM dosed (5 mg/kg iv) mice: solvent system 1-butanol–acetic acid–water (4:1:1).

Table III. Activity of AChE in Mice Brains Homogenates of a Control Group and of a Group Treated with DFP

Normal act.[a] × 10^6	Act.[a] × 10^6 (after sc injection of 2 mg/kg of DFP)
10.652	0.930
9.926	1.088
9.918	1.146
10.259	1.214
9.953	
10.983	1.332
Mean 10.248	1.142
SE ±0.198	±0.067

[a] Moles per liter per minute per gram of tissue.

this question, experiments were performed to study the dose and time dependence of the reactivation of AChE in mice brains inhibited by 0.5 LD_{50} of DFP (2 mg/kg subcutaneously). Table III lists the AChE activities of the control group of mice and that of a group of mice treated with DFP (2 mg/kg sc). This DFP dose was shown to inhibit 88.9% of the enzyme. Groups of mice were then administered the same dose of DFP and various iv doses of 2-PAM (30, 40, and 50 mg/kg) and pro-2-PAM (20, 30, 40, and 50 mg/kg), respectively. Table IV and Figure 2 summarize these experiments.

The possible effect of the "free" inhibitor released during homogenization could be neglected, because it was shown[28] to be present in very low concentrations in the case of DFP. The degree of reactivation was calculated according to the equation

$$\% \text{ reactivation} = (A_r - A_i)/(A_n - A_i) \times 100$$

where A_n = normal activity of AChE in brain, A_i = activity after intoxication, A_r = activity after intoxication and treatment with a reactivator, and % activity = A/A_n, where $A = A_r$ or A_i.

As Table IV and Figure 2 demonstrate, there is a dramatic difference between the effectiveness of 2-PAM to reactivate inhibited AChE in mice brains when it was given as such and when the pro-drug was administered. A relatively high dose of 2-PAM, 30 mg/kg, was still unable to reactivate any AChE, while pro-2-PAM even at 20 mg/kg resulted in an 18% reactivation of the blocked enzyme. Furthermore, extrapolation of the dose reactivation curve for pro-2-PAM shows (Figure 2) that full

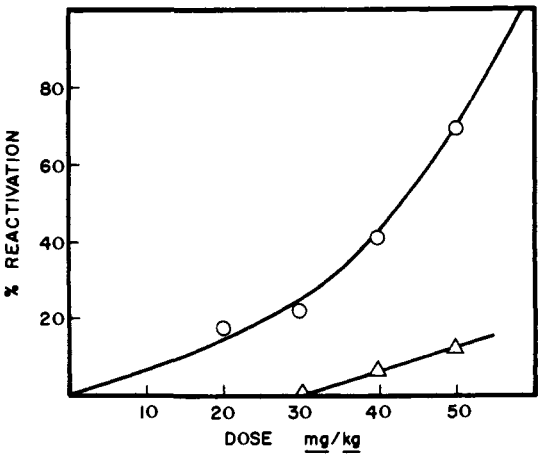

Figure 2. Fraction of in vivo reactivation of AChE in mice brains inhibited by 2 mg/kg of DFP (sc) vs. iv dose of 2-PAM (△) and pro-2-PAM (○), respectively.

Table IV. Activity and Reactivation of AChE in Mice Brain Given 2 mg/kg Sc of DFP and Treated 15 min Later with 2-PAM or Pro-2-PAM Given Iv

Drug	Dose, mg/kg	Act.[a] × 10^6	SE	% act.	% reactivation
2-PAM	30	1.053	0.076	10.27	0.00
2-PAM	40	1.683	0.041	16.42	5.93
2-PAM	50	2.246	0.187	21.92	12.12
Pro-2-PAM	20	2.781	0.163	27.14	18.00
Pro-2-PAM	30	3.111	0.063	30.36	21.63
Pro-2-PAM	40	4.871	0.355	47.53	40.95
Pro-2-PAM	50	7.429	0.183	72.49	69.04

[a] Moles per liter per minute per gram of tissue; activity values represent the mean of six mice in each dose group.

reactivation in the brain should be achieved with a dose lower than 60 mg/kg of pro-2-PAM.

The nonlinear dependence of the degree of reactivation upon the dose of pro-2-PAM is interesting. In order to try to explain this phenomenon, it must be kept in mind that after the iv injection, the degree of reactivation in CNS depends on two major processes: reaching the site of action and the reactivation itself. Thus, there are two primary possibilities. Assuming that penetration into the brain is linearly dependent on the dose, the curvature observed in Figure 2 might be attributed to the reactivation process itself. Nonlinear dependence of enzyme activity upon concentration of a reactivator or an inhibitor was shown[29] to indicate that more than one molecule of reactivator (or inhibitor) is participating in forming the complex with each active site on the enzyme. In the case of 2-PAM, it was earlier suggested[30,31] that the reactivator (R) forms a transition complex (EPhR) with the phosphorylated enzyme (EPh). This complex then breaks down to regenerate the enzyme E and the reaction product P.

$$\text{EPh} + \text{R} \rightleftarrows \text{EPhR} \rightarrow \text{E} + \text{P}$$

If more than one molecule of 2-PAM participates in forming the complex EPhR, then the dependency of the degree of reactivation upon the concentration of 2-PAM would be nonlinear. In the case of phosphorylated AChE (by isopropylmethylphosphorofluoridate) in vitro, the dependence of the fractional reactivation upon the concentration of 2-PAM was a sigmoid function.[32] Furthermore, since in in vivo experiments the source of the enzyme is not pure, one cannot exclude the possibility of a nonspecific binding of the reactivator to the protein.

On the other hand, it is possible that penetration of the pro-drug into the brain was "dose-dependent". It is clear that the amount of pro-drug available to diffuse through

the BBB is subject to the rate of oxidation especially in the blood stream. The slower the oxidation, the more pro-drug is expected to reach the brain. Thus, increasing the dose given directly in blood might saturate or partially saturate enzymes, such as dehydrogenases which are known to facilitate oxidation of amines.[33] This could result in higher pro-drug concentrations available for the brain. Another possibility for the nonlinear dependence is the eventual saturation of the active transport system of small cations out of the brain.[17]

The complexity of the system makes it impossible to decide which of the possibilities is really responsible for the observed nonlinear dependence.

It was found that although dialysis, dilution, and washing are ineffective in restoring the activity of a phosphorylated cholinesterase, the inhibited enzyme slowly recovers its activity on standing. The extent and rate of reversal depends upon inhibitor and enzyme type. However, the longer the inhibitor is in contact with the enzyme, the smaller is the fraction of the inhibited enzyme that can be recovered by a reactivating agent. This conversion to irreversibility is called "aging".[34] It was suggested that the rate-determining step in the aging reaction of methyl-phosphorylated AChE is a unimolecular fission of the C–O bond in the alkoxy leaving group.[35] This theory was substantiated by Michel et al.[36] Thus, the aging process with DFP could be represented as

$$\text{AChE} \xrightarrow{\text{DFP}} \text{AChE-O-P}(=\text{O})(\text{OC}_3\text{H}_7)(\text{OC}_3\text{H}_7) \xrightarrow{\text{"aging"}} \text{AChE-O-P}(=\text{O})(\text{OH})(\text{OC}_3\text{H}_7)$$

It was found[12] that DFP caused "aging" relatively fast (in a few hours). We have used the property of pro-2-PAM of penetrating the BBB for a complete in vivo demonstration of the "aging" process with DFP in the brain. Thus, the reactivation with 40 mg/kg of pro-2-PAM after various time intervals was determined in mice poisoned with 0.5 LD_{50} sc of DFP. The results are shown in Figure 3.

Despite the obvious aging process, by treating the mice with pro-2-PAM even 2 hr after intoxication, more reactivation could be achieved than by dosing with 2-PAM only 15 min after the intoxication. This demonstrates another possible advantage of the pro-drug relative to 2-PAM.

Experimental Section

Materials. 2-PAM chloride and phospholine iodide were obtained from Ayerst Laboratories, Inc. Acetylthiocholine iodide was supplied by Sigma Chemical Co. and 5',5'-dithiobis(2-nitrobenzoic acid) and DFP were obtained from Aldrich Chemical Co., Inc. Aquasol and [^{14}C]methyl iodide were supplied by New England Nuclear. [^{14}C]Methyl-labeled 2-PAM iodide (1 mCi/mmol) and [^{14}C]-pro-2-PAM chloride (0.65 mCi/mmol) were synthesized as described earlier.[1] Radioactivity counting was performed in a liquid scintillation system LS-150 (Beckman Instruments) using a known[37] method for preparation of biological materials.

Brain Level Studies. The radioactive 2-PAM and pro-2-PAM, respectively, were dissolved in the appropriate amount of citrate buffer, pH 3, and 0.1 ml of this solution was injected into the tail vein of white mice (ICR) weighing 30 ± 5 g. After 15 min the animals were decapitated; the brains were removed from the skull, washed with normal saline, and kept at -18° until they were prepared for counting. The brains were weighed and covered with 10 ml of 30% hydrogen peroxide for 3 hr. After homogenizing, an aliquot of 3 ml was mixed with 10 ml of Aquasol and counted.

Radiochromatography was performed using No. 1 Whatman paper strips 1 in. wide in a descending chromatographic chamber over 24 hr in a system of 1-butanol–acetic acid–water (4:1:1). The whole brain was homogenized in 5 ml of water. The homogenate was centrifuged at 35000 rpm. From the supernatant solution a protein-free solution was prepared and chromatographed. The strip was air-dried and was cut to 20 pieces each corresponding to a 0.05 R_f value. Each piece of paper was counted for radioactivity in a medium of 10 ml of Aquasol.

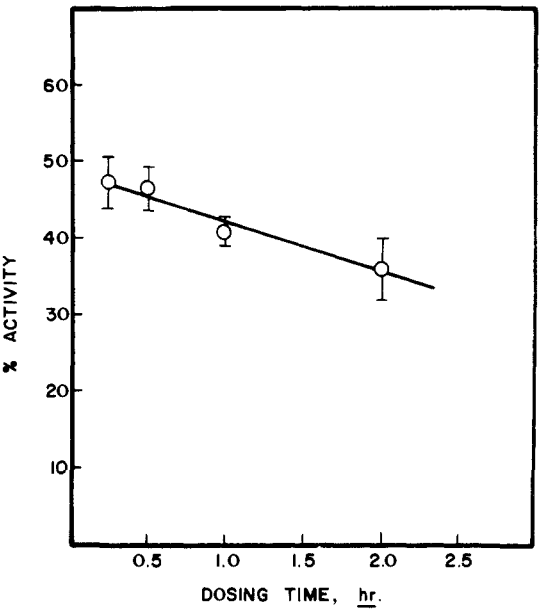

Figure 3. Activity of AChE in mice brains inhibited by 2 mg/kg of DFP (sc) and reactivated with 40 mg/kg of iv pro-2-PAM vs. time elapsed between the administration of the poison and of the antidote.

Reactivation of Phosphorylated Acetylcholinesterase in the Brain of Mice. White male mice (ICR), weighing 30 ± 5 g, were randomly divided into 12 groups, with six animals in each group. The control group was injected subcutaneously (sc) with normal saline and iv with 0.05 *M* pH 3 citrate buffer. Animals of the intoxicated untreated group received DFP, 2 mg/ml. Seven treated groups were injected sc with the same dose of DFP, and 15 min later they were injected iv with various doses, 20, 30, 40, 50 mg/kg of 2-PAM and pro-2-PAM, respectively, dissolved in 0.05 *M* pH 3 citrate buffer. Ninety minutes after the treatment the mice were decapitated, and the blood vessels in the brain were washed free of blood with normal saline before the removal of the brain from the cranial cavity. Brain homogenates were prepared in 25 ml of 0.1 *M* phosphate buffer, pH 8, using a motor driven Teflon on glass homogenizer.

AChE activity was measured by the method of Ellman et al.[38] using acetylthiocholine iodide as substrate and DTNB [5',5'-dithiobis(2-nitrobenzoic acid)] as chromogen. To 2.5 ml of 0.1 *M* pH 8 phosphate buffer were added 0.4 ml of the homogenate, 0.1 ml of 0.01 *M* DNTB solution, and 0.02 ml of 0.075 *M* substrate solution. The increase in the absorption at 412 nm was measured for 15 min by using a Cary Model 16 spectrophotometer. The reference cell included buffer, homogenate, and chromogen.

The three remaining groups of mice received the same dose of DFP and were treated iv with 40 mg/kg of pro-2-PAM at different time intervals, 30, 60, and 120 min after DFP dosing. After 90 min the mice were sacrificed and AChE activity in the brain was determined.

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Conformationally Restricted Analogs of Histamine H_1 Receptor Antagonists: *trans*- and *cis*-1-Benzyl-3-dimethylamino-6-phenylpiperidine†

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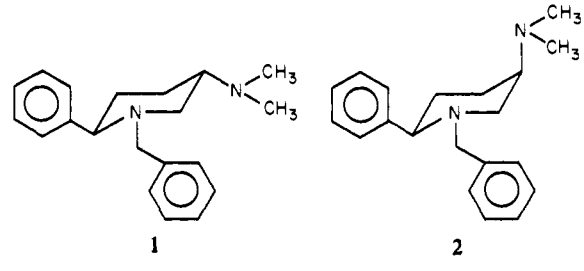
The syntheses of *trans*- and *cis*-1-benzyl-3-dimethylamino-6-phenylpiperidine (1 and 2) are described. Compounds 1 and 2 were found to be inhibitors of histamine, acetylcholine, and barium chloride induced contractions of the isolated guinea pig ileum. Compounds 1 and 2 do not exhibit appreciable stereoselectivity in their ability to inhibit smooth muscle contractions. The cis compound 2 is a more effective inhibitor of histamine *N*-methyltransferase than the trans isomer 1.

Recent reports from this laboratory have described the results of studies directed toward elucidating the stereochemical factors involved in the interaction of antagonists with histamine H_1 receptors.[1,2] Our approach has involved the use of conformationally restricted analogs of ethylenediamine histamine antagonists to ascertain the role of the conformation about the C–C bond of the dimethylaminoethyl group in determining antagonist activity. It was concluded from our earlier work that a fully extended trans N–C–C–N conformation is not necessary for H_1 receptor blockade by ethylenediamine antagonists and that the model proposed by Casy and Ison[3] represents a reasonable approximation of the molecular conformation of H_1 receptor-bound histamine antagonists.

The purpose of the present report is to describe the synthesis and pharmacological evaluation of *trans*- and *cis*-1-benzyl-3-dimethylamino-6-phenylpiperidine (1 and 2). These compounds are cyclic analogs of the ethylenediamine histamine antagonists and they contain the essential structural features usually associated with antagonist activity.[4] Compounds 1 and 2 were selected for study in order to further assess the importance of conformational factors in determining the effectiveness of antagonist–receptor interaction. The trans compound 1 would be expected to exist almost exclusively in the conformation in which the 3-dimethylamino group and the 6-phenyl group are equatorial. In this conformation, the N–C–C–N dihedral angle is 180°. On the other hand, compound 2 would be expected to exist as an equilibrium mixture of the axial and equatorial 3-dimethylamino conformers with only the equatorial 3-dimethylamino, axial 6-phenyl conformer, or a boat conformation, exhibiting the 180° N–C–C–N arrangement. Therefore, if molecular



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† This work is dedicated to the memory of Edward E. Smissman.