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NEUROTOXICITY OF N-(2-CHLOROETHYL)-N-ETHYL-2-BROMOBENZYLAMINE HYDROCHLORIDE (DSP 4) ON NORADRENERGIC NEURONS IS MIMICKED BY ITS CYCLIC AZIRIDINIUM DERIVATIVE

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The haloalkylamine N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP 4), impairs the ability of central and peripheral noradrenergic neurons to take up exogenous noradrenaline (NA) and produces a long-lasting reduction of endogenous NA levels. Previous work has shown that DSP 4 apparently binds to the NA carrier system where it is cyclized spontaneously to an aziridinium compound that seems to trigger the degenerative changes responsible for NA depletion as a result of the alkylation and irreversible inactivation of the carrier. To establish the importance of the binding of unchanged DSP 4 for the production of these changes, the compound was injected immediately after dissolving it or after its incubation under conditions known to favor its conversion into the aziridinium derivative. Endogenous NA levels were studied in the brain and heart of adult mice. DSP 4, given immediately after being dissolved, depleted heart and brain NA. When injected after being incubated, DSP 4 reduced NA levels in the periphery but not in the central nervous system. This failure was due to the inability of the aziridinium ion to pass the blood-brain barrier because it could deplete NA when given directly into the brain. Pretreatment with the uptake blocker desmethylimipramine counteracted the effects of both DSP 4 and the aziridinium derivative. Thus, fixation of DSP 4 to the carrier is not a prerequisite for the activity of the aziridinium derivative as the derivative can interact directly with the membrane NA uptake system of central and peripheral noradrenergic neurons to produce the changes characteristic of DSP 4 administration.

Noradrenergic neurons Noradrenaline DSP 4 neurotoxin Aziridinium ion

1. Introduction

It has been shown recently that the tertiary haloalkylamine, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP 4) produced long-lasting changes in central and peripheral noradrenergic neurons of rodents. The compound permanently impairs the uptake of exogenous noradrenaline (NA) by brain tissue slices or synaptosomes and markedly depletes endogenous NA from several brain regions. In the periphery, similar alterations in neuronal function are found in sympathetically innervated organs but in this case there is a gradual recovery with a return to normality after approximately 2 weeks (Ross et al., 1973; Ross, 1976; Ross and Renyi, 1976; Jonsson et al., 1978; Kammerer et al., 1979; Jaim-Etcheverry and Zieher, 1980). Moreover, DSP 4 given perinatally interferes with the ontogenetic development of central noradrenergic neurons (Jaim-Etcheverry and Zieher, 1980). Thus, the changes produced by DSP 4 in mature and in developing noradrenergic neurons are very similar to the well known neurotoxic effects of 6-hydroxydopamine (6-OHDA) and of its precursor amino

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acid 6-hydroxydopa (6-OHDOPA) (for review see Thoenen and Tranzer, 1973; Kostrzewa and Jacobowitz, 1974). DSP 4 which also shows specificity for noradrenergic neurons (Ross, 1976; Jonsson et al., 1978; Kammerer et al., 1979), has the advantage of being active in the central nervous system after parenteral administration because, as a tertiary amine, it easily overcomes the blood-brain barrier.

The target of DSP 4 action seems to be the carrier system responsible for NA transport into the neuron. Previously published data show that DSP 4, a tertiary amine, cyclizes spontaneously to generate a quaternary ammonium derivative. Experiments by Ross et al. (1973) demonstrated that in aqueous media and at physiological pH, DSP 4 was converted 'in vitro' into an aziridinium compound (fig. 1) with a half life of 7 min. From his studies on the pharmacology of this compound, Ross (1976) has suggested that this alkylating derivative formed at the site of DSP 4 binding would be responsible for the long-lasting impairment of the NA uptake process which in turn causes neuronal degeneration and ensuing depletion of NA. There are examples of compounds whose pharmacological actions are due to the generation of aziridinium ions and subsequent alkylation of biological structures. Blockers of α-adrenergic receptors such as dibenamine and phenoxybenzamine, which have a 2-haloethyl group and are thus related to DSP 4, also cyclize into an active aziridinium compound under conditions that have been carefully characterized (Nickerson and Gump, 1949; Graham, 1957; Krueger and Cook, 1975; Henkel et al., 1976).

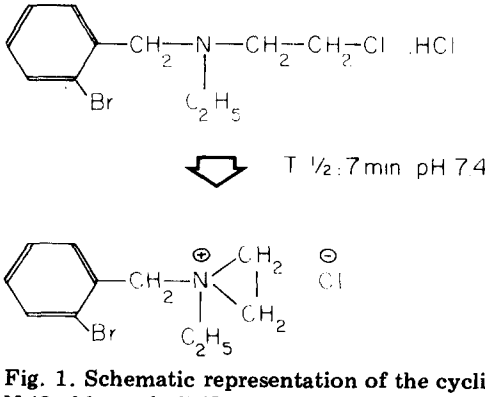

Fig. 1. Schematic representation of the cyclization of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP 4) to its aziridinium derivative (according to Ross et al., 1973).

In order to determine if the initial fixation of DSP 4 to the carrier system is necessary for the long-lasting depletion of NA or if, alternatively, the aziridinium ion is the active species and is capable of causing depletion directly, we studied NA levels in central and peripheral noradrenergic neurons of adult mice injected with DSP 4 immediately after it was dissolved or after different periods of incubation under conditions shown to generate the cyclic derivative (Ross et al., 1973; Krueger and Cook, 1975). The latter was found to be as active as DSP 4 in depleting heart NA and, although unable to cross the blood-brain barrier, reduced brain NA when given intraventricularly. These effects seem to take place at the level of the neuronal membrane.

2. Materials and methods

2.1. Treatment of animals

Female albino mice of the BALBc strain weighing 25—30 g were used in these experiments. N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP 4) was synthesized by Dr. Richard Dahlbom and collaborators, University of Uppsala, Sweden. In several groups of experimental animals, the compound was injected at a dose of 50 mg/kg i.p. after dissolving it in distilled water or in 0.05 M sodium phosphate buffer pH 7.4. Injections were made either immediately after dissolving DSP 4 or after incubation of DSP 4 solutions in buffer for varying periods at 37° C in a water bath. The cyclized derivative of DSP 4 is formed at this pH and temperature. To determine the effects of DSP 4 given directly into the brain, a group of mice received different doses of DSP 4 ranging

from 15 to 100 μg (expressed as the free base) dissolved in 5 μl of water or buffer and injected into the lateral ventricle immediately after dissolving it or after incubation as described above (Noble et al., 1967). In some animals, the amine uptake blocker desmethylimipramine (DMI, Pertrofran, Ciba-Geigy) was given i.p. at a dose of 15 mg/kg, 20 min before the systemic or the intraventricular injection of DSP 4. Control animals received the corresponding diluents, water or buffer, at the same time intervals.

2.2. Tissue sampling and noradrenaline assay

Mice were decapitated 48 h after injections unless otherwise indicated. The brain was exposed and separated by a cut at the caudal tip of the cerebellum. The cerebellum as well as the olfactory tubercles and the pineal gland were discarded. NA was extracted from the whole brain and from the heart in 0.4 N perchloric acid containing 0.2% EDTA and 0.005% $Na_2S_2O_5$. NA was isolated from these extracts by cation column exchange chromatography (Bertler et al., 1958) and its content in the eluates from the columns was determined fluorimetrically (Häggendal, 1963). The results were not corrected for recovery which was 89 ± 3.2%. The significance of differences between values was determined by means of Student's t-test.

3. Results

3.1. Effects on brain and heart NA levels of DSP 4 injected immediately after being dissolved or after incubation

NA levels were reduced in mouse brain and heart by 62% and 70% respectively 48 h after the i.p. injection of 50 mg/kg DSP 4 dissolved in water immediately before administration. A similar reduction was produced by DSP 4 given immediately after being dissolved in phosphate buffer pH 7.4 (fig. 2).

To establish whether the cyclized derivative

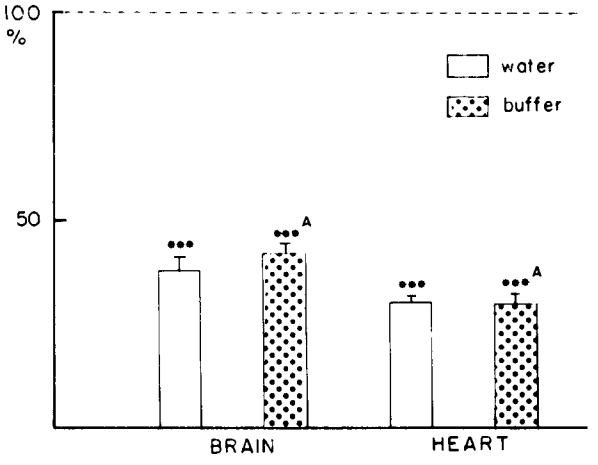

Fig. 2. Modifications in the content of endogenous NA in the brain and heart of mice injected with DSP 4 (50 mg/kg i.p.) immediately after dissolving the DSP 4 in either water (open bars) or phosphate buffer pH 7.4 (shaded bars). Animals were killed 48 h after the injection. Results are expressed as percentages of untreated control values. Absolute control values for NA (ng/g weight): brain 198 ± 6; heart 825 ± 14. Each value represents the mean ± S.E.M. from 3—5 groups of 5—7 mice each. *** $P < 0.001$ when compared with controls; [A] not significantly different from values obtained after injection of DSP 4 dissolved in water.

of DSP 4 formed by incubating DSP 4 at pH 7.4 at 37°C, had effects similar to those of the parent compound, NA levels were determined in the brain and the heart of mice killed 48 h after the i.p. injection of 50 mg/kg of DSP 4 either just dissolved in buffer or first incubated for various periods at 37°C. Fig. 3 shows that by prolonging the incubation in buffer before injection, the ability of DSP 4 to deplete brain NA was progressively reduced. The depletion of brain NA 48 h after treatment, was maximal when DSP 4 was dissolved and injected immediately. Brain NA depletion was gradually less marked after incubation of DSP 4 for 10 or 21 min and finally, when incubated for 42 or 70 min before injection, DSP 4 did not modify NA levels in the brain.

As is also shown in fig. 3, DSP 4 dissolved in buffer and immediately injected, produced a 70% depletion of heart NA after 48 h. When

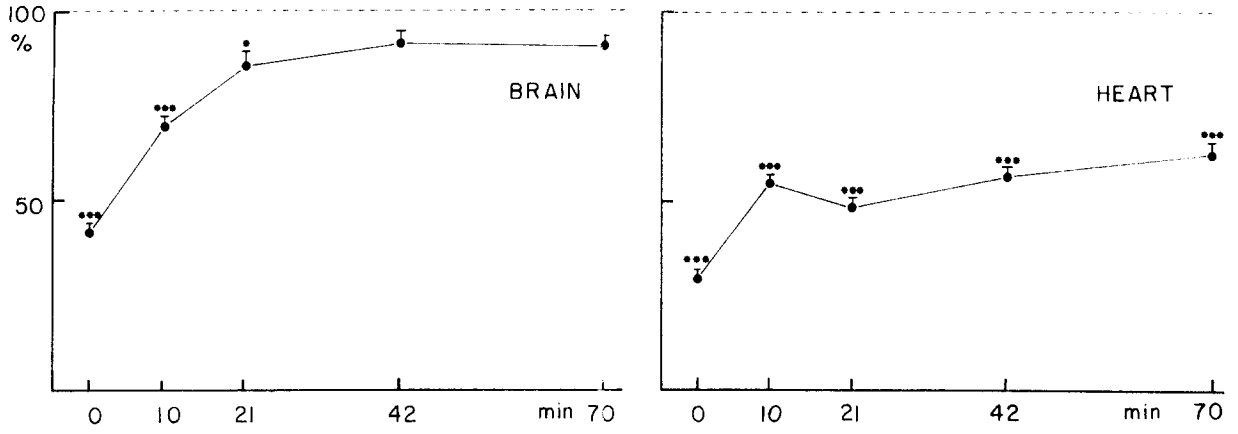

Fig. 3. Modifications in the content of endogenous NA in the brain and the heart of mice injected with DSP 4 (50 mg/kg i.p.) either immediately after dissolving it in phosphate buffer pH 7.4 (time 0) or after various periods of incubation at 37°C. Animals were killed 48 h after the injections. Results are expressed as percentages of untreated control values. Absolute control values for NA (ng/g weight): brain 175 ± 5; heart 785 ± 13. Each value represents the mean ± S.E.M. from 4—6 groups of 6—8 mice each. * $P < 0.05$; *** $P < 0.001$ when compared with controls.

DSP 4 was incubated for various periods prior to injection (10—70 min), it produced a significant depletion of heart NA, ranging from 39 to 52% as compared to control values. These effects were significantly smaller than those observed after the immediate injection of DSP 4.

3.2. Brain noradrenaline levels after DSP 4 injected intraventricularly immediately after being dissolved or after being incubated at 37°C

To find if the lack of effect of the cyclized derivative generated from DSP 4 after incubation on brain NA was due to its inability to cross the blood-brain barrier, brain NA levels were studied 48 h after the intraventricular injection of several doses of DSP 4 given either immediately after dissolving it or after its incubation for 42 min at 37°C in phosphate buffer pH 7.4. As already discussed, the systemic injection of DSP 4 after such incubation did not modify NA levels in the brain.

Fig. 4 shows that 48 h after the intraventricular injection of 5 μg, 15 μg or 50 μg or DSP 4, brain NA levels were reduced to the same extent whether the compound was dissolved and injected immediately or was first incubated. The maximal depletion was obtained after the injection of 100 μg of DSP 4 immediately after it had been dissolved. However, it was not possible to determine the effects produced by the injection of 100 μg of DSP 4 after incubation because in this case, all mice died during the following 24 h. The toxicity of the compound was increased by incubation at all dose levels tested. The mortality of mice injected intraventricularly with the compound after its incubation, exceeded the mortality after the injection of DSP 4 just dissolved.

To establish if the effects produced by the intraventricular injection of DSP 4 were long-lasting, brain NA levels were determined in mice which received an intraventricular injection of 50 μg of DSP 4 immediately after being dissolved, 30 days before killing. Brain NA was depleted by 36% with respect to the controls. Absolute values for NA were (ng/g weight): 250 ± 5 (controls) and 158 ± 13 (DSP 4), the difference between these values being statistically significant ($P < 0.001$). This long-term depletion of brain NA, which was also found after injecting incubated DSP 4, was similar to

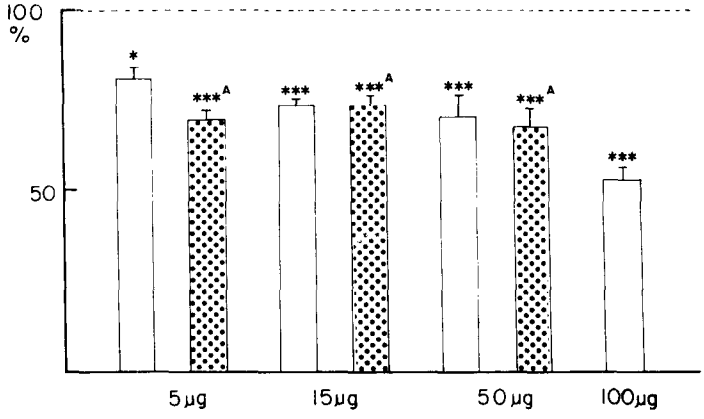

Fig. 4. Modifications in the content of endogenous NA in the brain of mice injected 48 h after intraventricular injection of various doses of DSP 4. The compound was dissolved in water and injected immediately (open bars) or dissolved in phosphate buffer pH 7.4 and incubated for 42 min at 37°C (shaded bars). Results are expressed as percentages of untreated control values. Absolute control values for brain NA (ng/g weight): 211 ± 4. Each value represents the mean ± S.E.M. from 3—4 groups of 4—6 mice each. * $P < 0.005$; *** $P < 0.001$ when compared with controls; [A] not significantly different from values obtained after injection of DSP 4 immediately after being dissolved.

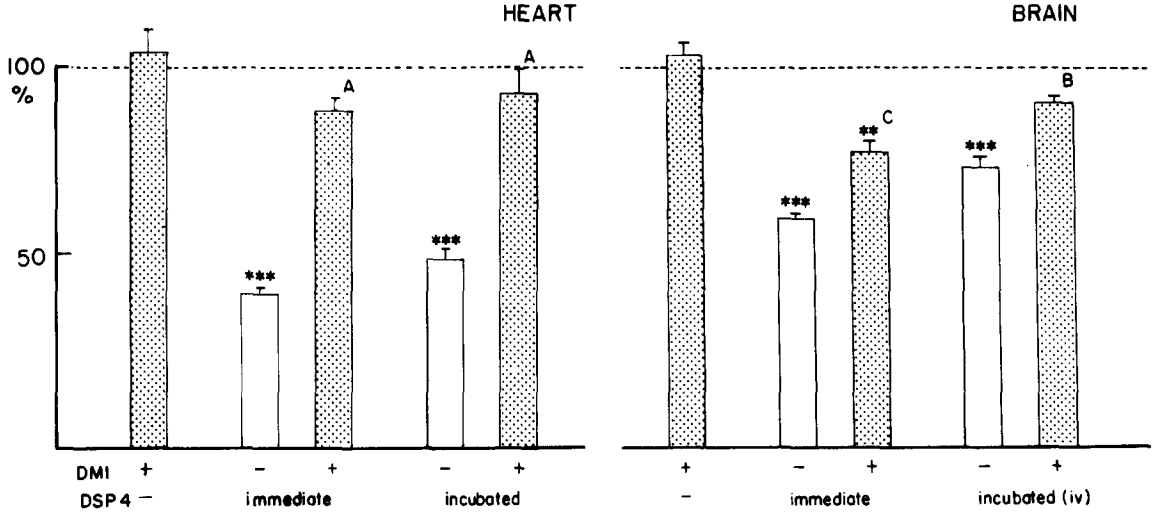

Fig. 5. Effects of pretreatment with desmethylimipramine (DMI, 15 mg/kg i.p., shaded bars) on the modifications of heart (left) and brain (right) NA levels produced by the injection of DSP 4 either immediately after dissolving it in water or after incubation for 42 min at 37°C in phosphate buffer pH 7.4. Mice received 50 mg/kg DSP 4 i.p. either immediately after it had been dissolved or after its incubation in buffer. Since DSP 4 did not modify brain NA levels when injected systemically after incubation, 15 μg of the incubated compound were injected intraventricularly (i.v.) to study the DMI-induced modifications of the NA depleting activity of incubated DSP 4. DMI was given 15 min before DSP 4 and the mice were killed 48 h after treatment. The results are expressed as percentages of untreated control values. Absolute control values for NA (ng/g weight) were: heart 832 ± 27; brain 225 ± 7. Each value represents the mean ± S.E.M. from 3—4 groups of 5—7 mice each. *** $P < 0.001$; ** $P < 0.01$ when compared with control values; [A] $P < 0.001$ and [B] $P < 0.01$ when compared with DSP 4 alone and not significantly different from controls or DMI alone; [C] $P < 0.001$ when compared with DSP 4 alone and with DMI alone.

that observed 48 h after the injection of the same dose of DSP 4.

3.3. Effect of desmethylimipramine on the depletion of NA from heart and brain produced by DSP 4 injected immediately or after incubation

To find if blockade of the NA transport mechanism modified the depletion of NA produced by DSP 4, DMI was injected at a dose of 15 mg/kg i.p. 20 min before treatment with DSP 4. The latter was given at a dose of 50 mg/kg i.p., either immediately after being dissolved in phosphate buffer pH 7.4 or after incubation at 37°C for 42 min. As already shown, the systemic injection of DSP 4 after 42 min incubation did not modify NA levels in the brain. Thus, in order to be able to study the effects of DMI on the depletion of brain NA by incubated DSP 4, DSP 4 was injected intraventricularly (15 μg in 5 μl). Fig. 5 shows NA levels in the heart and the brain of mice thus treated 48 h before killing. DMI per se did not modify the heart NA content but effectively prevented the depletion of NA produced by DSP 4 either dissolved and injected immediately or given after being incubated. In the brain, DMI did not modify endogenous NA levels but it also prevented significantly the depletion of NA produced by DSP 4 injected systemically after it had been dissolved. However, this protection was less marked than in the heart (Ross, 1976). The depletion of brain NA produced by the intraventricular injection of incubated DSP 4 was counteracted by DMI pretreatment.

4. Discussion

Endogenous NA was markedly depleted from the heart and brain of adult mice 48 h after the injection of DSP 4 immediately after being dissolved. Previous studies have shown that in the periphery the altered parameters recover and return to normal within 2 weeks while the impairment of noradrenergic neurons in the central nervous system seems to be permanent (Ross et al., 1973; Ross, 1976; Ross and Renyi, 1976; Jonsson et al., 1978; Jaim-Etcheverry and Zieher, 1980). When solutions of DSP 4 are incubated at neutral pH and at 37°C, there is a spontaneous and time-dependent conversion of the compound to its corresponding aziridinium derivative (Ross et al., 1973; Krueger and Cook, 1975). The half life of DSP 4 in such a system in vitro is 7 min (Ross et al., 1973). Although the depletion of NA was maximal 48 h after the injection of 'dissolved' DSP 4, the injection of solutions of the compound incubated for different periods of time produced marked reductions in heart NA levels, irrespective of the duration of the incubation prior to injection. In the brain however, the incubation time had a marked influence on the effects on NA levels. With increasing incubation times, the capacity of the injected compound to reduce NA levels was progressively lost. This lack of effect on brain NA of DSP 4 solutions injected after incubation for 42 min or more, suggested that the incubated solution contained a compound that was active, as shown by the effectiveness of the very same solutions in reducing heart NA, but unable to penetrate into the brain. If correct, this explanation would agree with the proposed generation in the solution of the aziridinium derivative, a quaternary ammonium compound unable to cross the blood-brain barrier (Ross et al., 1973).

To test this hypothesis, the barrier was bypassed by injecting either 'dissolved' or 'incubated' DSP 4 into the lateral ventricle. Incubation was for 42 min, a time at which DSP 4 solutions lost their ability to deplete brain NA after systemic injection. The finding that under these conditions DSP 4 had almost similar effects on brain NA both before and after incubation indicates that the aziridinium derivative is also effective in depleting NA from central noradrenergic neurons. It also confirms that its lack of central action after systemic injection, when it is very active in reducing peripheral NA, is due to its inability to pass the blood-brain barrier. As is the case

when DSP 4 is given systemically (Ross, 1976; Jaim-Etcheverry and Zieher, 1980), the compound produces a long-term depletion of brain NA after intraventricular injection. Similar concentrations of NA were found in the brains of mice killed 2 days or 30 days after injection. Interestingly, the intraventricularly injected compound was more toxic after incubation, probably due to other actions of the extremely reactive alkylating derivative.

As has been already mentioned, the primary effect of DSP 4 is thought to be irreversible damage of the NA carrier system located in the neuronal membrane. This assumption is favored by the finding that blockers of NA uptake such as cocaine and DMI antagonize both the long term impairment of NA uptake as well as the depletion of NA produced by β-substituted benzylamines (Ross, 1976; Ross and Renyi, 1976, Jonsson et al., 1978; Kammerer et al., 1979). In this study, DMI pretreatment protected heart NA from the depleting effect of both DSP 4 and the aziridinium derivative generated by incubating solutions of the parent compound. DMI pretreatment also blocked the brain NA depletion produced by DSP 4 given systemically immediately after dissolving it although the block was not as complete as in the case of the heart. The uptake blocker was also effective in counteracting the depletion of brain NA produced by the intraventricular injection of incubated DSP 4. Taken together, these observations indicate that the blockade of the NA transport system prevents the depletion of NA produced not only by DSP 4 but also by its aziridinium derivative in both peripheral and central noradrenergic neurons. This depletion might require the intraneuronal accumulation of DSP 4 as in the case of 6-OH-DA but there are data suggesting that such intraneuronal accumulation of DSP 4 is not a prerequisite for the depletion of NA (Ross, 1976). Moreover, the activity shown by the quaternary ammonium compound, an ionic species incapable of penetrating easily through cell membranes (Henkel et al., 1976) as well as the blockade of its effects by DMI, support the idea that the initial changes triggering the long-lasting modifications of noradrenergic neurons most probably take place at the external side of the neuronal membrane.

Thus, as is the case for the blockade of α-adrenergic receptors by haloethylamines structurally related to DSP 4 such as dibenamine and phenoxybenzamine, the depletion of NA in peripheral and central noradrenergic neurons by DSP 4 seems to depend on the presence of the aziridinium derivative rapidly and spontaneously generated under conditions prevailing in biological fluids. This alkylating compound would react covalently with anionic components of the uptake site, probably those responsible for attracting the cationic amino group of NA, thus causing competitive irreversible inhibition of NA uptake (Ross, 1976; Kammerer et al., 1979). It seems that the NA carrier can fix the aziridinium compound directly and that a selective fixation of the parent molecule, DSP 4, to this site is not a prerequisite for initiating the chain of events leading to the long-lasting changes in noradrenergic neurons. However, the effects in peripheral neurons may be slightly more potent when the parent amine is initially fixed to the carrier as suggested by the somewhat greater depletion of heart NA produced by the injection of 'dissolved' DSP 4 (70%) in comparison with the reductions obtained when the incubated solutions were injected (averaging 45%). This was not confirmed in the case of brain NA because similar depletions were produced by DSP 4 whether it was injected immediately or after incubation. Differences are thought to exist between the affinities of the compound for the NA carrier in central and in peripheral neurons (Ross, 1976).

In conclusion, the cyclic azirdinium derivative spontaneously generated from DSP 4 seems to be responsible for the effects of the latter because it depletes NA in the periphery in the same way as in the brain. In this case, it only acts when given directly into the central nervous system due to its inability to cross the blood-brain barrier. When injected imme-

diately after being dissolved, the tertiary amine DSP 4 would pass the barrier and undergo the intramolecular cyclization process within the brain. With increasing knowledge of their actions, these compounds are emerging as interesting tools in the study of the biology of noradrenergic neurons in adult and developing rodents.

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