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STEREOSPECIFICITY OF ANTIDOTES AND THEIR MECHANISM OF
ACTION IN INTOXICATIONS WITH ORGANOPHOSPHORUS
ANTICHOLINESTERASES

ANNUAL REPORT

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19. ABSTRACT

The following effects of diazepam on ACh dynamics and binding of l-hyoscyamine were registered:

1. A small increase of brain ACh. A large increase of brain Ch.
2. Reduced uptake and elimination of deuterated Ch in brain. The effect was specific for Ch; in comparison, the kinetics of cotinine, used as a model substance for passive diffusion across the blood-brain barrier, were unaffected by diazepam.
3. Increased clearance of Ch from blood.
4. Prevention and reversal of OT-induced tremor but not hypothermia.
5. Decreased l-hyoscyamine binding.

The effects of diazepam on the ACh dynamics are consistent with diazepam's known potentiation of gamma-aminobutyric acid's inhibitory function in nerve transmission, with a decreased turnover rate of ACh and increased levels of ACh and Ch as results. The modulating effect of diazepam on the binding properties of muscarinic receptors is probably one of the mechanisms responsible for its profound effects in treatment of intoxications with anticholinesterases. *Keywords: Antidotes, Cholinesterase inhibitors. (A)*

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SUMMARY

In cases of organophosphate intoxications, the addition of diazepam to the conventional atropine-oxime treatment has been shown to improve the prophylaxis and therapy. This prompted us to study the effect of diazepam itself on the acetylcholine (ACh)-synthesizing system in mouse brain in vivo. ACh and choline (Ch) were analyzed by gas chromatography-mass spectrometry using deuterated internal standard. Turnover of ACh was studied by following the incorporation of Ch into ACh after i.v. injection of [$^2\text{H}_6$]Ch. Diazepam was found to increase endogenous levels of ACh and Ch and decrease turnover rate. The most pronounced effects were the elevated endogenous Ch levels and a smaller amount of deuterated [$^2\text{H}_6$]Ch reaching the brain. A possible explanation for these findings is that diazepam affects the Ch transport across the blood-brain barrier. In experiments in which levels of endogenous and $^2\text{H}_6$ -labelled Ch were analyzed in blood following i.v. injection of the latter, [$^2\text{H}_6$]Ch was eliminated faster in diazepam-treated animals, and the increased level of endogenous blood Ch returned more rapidly to normal, indicating an increased capacity to eliminate blood Ch. Experiments in which [$^2\text{H}_6$]Ch was injected 1 min before diazepam indicated that elimination of Ch from brain was affected by diazepam. To elucidate whether the effect of diazepam on uptake and elimination of brain Ch is a general effect or is specific for Ch, we studied the effect of diazepam on the uptake and elimination of cotinine, a tertiary amine which is cholinergically inactive. Diazepam did not influence the kinetics of cotinine, which led us to believe that the effect is specific. Diazepam prevented oxotremorine (OT)-induced tremor when injected both before and after the OT administration. Tremor is elicited by the muscarinic effects of OT. Diazepam did not prevent OT-induced hypothermia.

When studying the mechanism of action of a drug in the cholinergic nervous system, new insight may be gained by measuring changes in the size of the functional muscarinic receptor pool. We have developed a technique that allows such studies to be performed in vivo under physiological conditions. By separate injection of the optical antipodes of

atropine, d- and l-hyoscyamine, in mice and following their kinetics in different parts of the brain, it was possible to separate the specific receptor binding of the "active" antipode l-hyoscyamine from the unspecific binding of the "inactive" antipode d-hyoscyamine. The concentration of the antipodes was measured by gas chromatography-mass spectrometry and deuterated internal standard. The concentration of specifically bound l-hyoscyamine is assumed to correspond to the size of the functional muscarinic receptor pool. The physiological significance of this concentration of l-hyoscyamine was confirmed by its blocking effect on OT-induced tremor. By using this technique, diazepam was found to decrease the functional muscarinic receptor pool.

One of the mechanisms responsible for the profound influence of diazepam on the effect of anticholinesterases is probably its modulating effect on the binding properties of muscarinic receptors. Presynaptic receptor modification may lead to the effects on the dynamics of Ch and ACh. The decrease of ACh turnover might play a role in the antidotal effect on organophosphate intoxications.

FOREWORD

Since the execution of this grant was delayed for a considerable length of time, the research outlined in the proposal was commenced before the grant was approved. Therefore, this report covers work carried out during the entire period of time spent on the project.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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INTRODUCTION

The use of diazepam in the prophylaxis and therapy of organophosphate intoxications is now well documented (1,2). Yet the mechanism for the remarkable potentiation of the antidotal effect achieved when adding diazepam to the conventional atropine-oxime therapy remains unclear and raises questions about a possible link with the cholinergic system, direct or indirect, for diazepam. Since diazepam acts by enhancing the inhibiting effect of gamma-aminobutyric acid (GABA) on nerve transmission, it should be possible to demonstrate its effect in organophosphate intoxication via the dynamics of acetylcholine (ACh). Previous reports have demonstrated an effect of diazepam on endogenous levels of ACh, but not choline (Ch), in striatum and hippocampus of rats (3,4), and an inhibited release of ACh (5). Metlas et al. (6), however, found that in synaptosomes from the brain of diazepam-treated rats the synthesis and release of ACh were unaffected by the drug, but the accumulation of [3 H]Ch was diminished. This prompted us to study the effects of diazepam on the ACh-synthesizing system in mouse brain, as well as on the size of the functional muscarinic receptor pool. We also studied the pharmacological effect of diazepam on symptoms induced by the muscarinic agonist oxotremorine (OT), e.g., tremor, hypothermia, salivation, and diarrhea.

Studies on muscarinic receptor concentration and comparative binding assays of agonists and antagonists are mostly done in vitro by incubation and measurement of binding to tissue homogenates of radiolabelled potent antagonists, e.g., [3 H]scopolamine or [3 H]quinuclidinyl benzilate, according to Yamamura and Snyder (7). We have developed a technique that allows studies of the receptor concentration to be performed in vivo under physiological conditions. Hypothetically, the optical antipodes of atropine, d- and l-hyoscyamine, exhibiting great differences in muscarinic receptor antagonistic properties, would bind differently to such receptors. By injecting the two antipodes separately and following their kinetics in brain, it was possible to separate specific receptor binding of

the "active" antipode l-hyoscyamine from that of the "inactive" antipode d-hyoscyamine, representing nonspecific binding. The physiological relevance of this low concentration of specifically bound l-hyoscyamine has been assessed by studying its blocking effect on tremor induced by OT.

MATERIALS AND METHODS

Animals

Male NMRI mice weighing 20-25 g were used.

Chemicals

Deuterium-labelled ACh and Ch were synthesized according to Karlén et al. (8). Diazepam was in the form of Diazemuls (KabiVitrum AB, Sweden). The reagents used for analysis of ACh and Ch were prepared as described by Karlén et al. (8). l- and d-Hyoscyamine were a gift from Prof. P. Waser, Zurich, Switzerland. The melting point of the two substances were 108-109°C and 105-106°C, respectively, which corresponds well to the published values in CRC, Handbook of Chemistry and Physics (1970) (108.5°C and 106°C). The synthesis of deuterium-labelled atropine is described in Palmér et al. (9). Oxotremorine oxalate, 1-(2-oxo-1-pyrrolidinyl)-4-(1-pyrrolidinyl)-2-butyne, was synthesized according to Karlén and Telc (10). All other chemicals were of analytical grade.

Drugs and their administration

Diazepam (Diazemuls) was given in doses of 1 mg/kg i.v. or 2 mg/kg i.p. l- or d-Hyoscyamine (base) was given i.v. in doses of 1, 2, or 4 mg/kg. Oxotremorine (oxalate) was injected i.v. in doses of 0.1 or 0.5 mg (base)/kg. Cotinine was given i.v. in a dose of 2 mg/kg. The drugs were dissolved in, or diluted with, saline and given in a volume of 5 ml/kg.

Analysis of ACh and Ch in mouse brain

ACh and Ch were analyzed using gas chromatography-mass spectrometry (GC-MS) and deuterated internal standards, according to Karlén et al.

(8). The mice were killed by focused microwave irradiation on the head with 2.5 kW for 0.68 sec (Metabostate, Gerling-Moore, Palo Alto, CA) to inactivate enzymes rapidly and to prevent postmortem changes of ACh and Ch. The brain was homogenized in 4 ml 0.4 M HClO_4 . $[\text{}^2\text{H}_9]\text{ACh}$ and $[\text{}^2\text{H}_9]\text{Ch}$ were added as internal standards. The homogenates were centrifuged for 20 min at $100,000 \times g$. ACh and Ch together with their deuterated moieties were extracted into methylene chloride as ion pairs with dipicrylamine (2,4,6,2',4',6'-hexanitrodiphenylamine) (DPA) and then demethylated with sodium benzenethiolate to form the corresponding tertiary amines, which were analyzed by GC-MS using a 25-m DB1701 capillary column at 180°C .

Blood was collected in tubes containing 0.4 M HClO_4 and analyzed for Ch as described for brains.

Turnover of ACh in mouse brain

The ACh turnover (TR_{ACh}) in brain was studied by following the incorporation of $[\text{}^2\text{H}_6]\text{Ch}$ into ACh after an i.v. injection of $[\text{}^2\text{H}_6]\text{Ch}$ (20 $\mu\text{mol/kg}$), in a volume of 5 ml/kg administered during 1 sec. The turnover rate of ACh was calculated from the specific activities of ACh (S_{ACh}) and Ch (S_{Ch}) 15 and 45 sec after the $[\text{}^2\text{H}_6]\text{Ch}$ injection, according to Zilversmit (11), as described by Karlén et al. (12) and Nordgren et al. (13).

Analysis of d- and l-hyoscyamine

The mice were killed by focused microwave irradiation as described above. The brain tissue (cortex, cerebellum, striatum, hippocampus, or in some cases half the brain excluding cerebellum) was homogenized in 2 ml 0.4 M HClO_4 containing $[\text{}^2\text{H}_3]\text{atropine}$ as internal standard. The homogenates were centrifuged for 20 min at $100,000 \times g$. The concentrations of d- and l-hyoscyamine were determined according to Palmér et al. (9) and Olsson et al. (14) with the following modifications: to the super-

nantant 0.25 ml 5 M NaOH and 6 ml diethyl ether were added. After agitation and centrifugation the ether layer was evaporated to dryness. Thirty microliters of N,O-bis(trimethylsilyl) acetamide (BSA) reagent was added to the residue and after reaction for 30 min at 60°C, the excess of reagent was evaporated. The residue was dissolved in 25 µl methylene chloride and analyzed by GC-MS. The gas chromatographic column used was a 12-m SE-52 fused silica capillary column. Column temperature was 210°C.

Analysis of cotinine in mouse brain

The mice were killed by focused microwave irradiation as described above. The brain was homogenized in 0.4 M HClO₄ containing the internal standard, [²H₂]cotinine. The homogenates were centrifuged for 20 min at 100,000 × g. After alkalization of the supernatant and extraction with methylene chloride, the organic phase was dried with sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in methylene chloride and analyzed by GC-MS using a 15-m SP-1000 capillary column at 200°C.

Recording of tremor

A mouse is placed on a plastic air cushion. The movements of the mouse are transferred from the air cushion via a plastic tube to a pressure transducer (Model PT5, Grass Instrument Co., Quincy 79, MA), where it is converted into a low frequency a.c. voltage. This voltage is amplified and filtered through a bandpass filter tuned to the frequency of the tremor (11-28 Hz). The bandpass-filtered tremor analogue is rectified and fed into a second order lowpass filter with a time constant of 0.2 sec: Hereby a continuous moving time average of tremor activity is accomplished. The area under the curve is the total activity for a given time. For recording apparatus, see block diagram (Fig. 1).

Temperature measurement

The body temperature ($^{\circ}\text{C}$) was measured using a rectal thermometer (Tele-thermometer 43 TA, Yellow Springs Instrument Co., Yellow Springs, OH).

RESULTS AND DISCUSSION

Effect of diazepam on oxotremorine-induced tremor

Diazepam (1 mg/kg, i.v.) injected 30 sec prior to OT (0.5 mg/kg, i.v.) effectively prevented the induced tremor. It also counteracted the tremor when administered after OT (Fig. 2). l-Hyoscyamine, the pharmacologically "active" antipode of atropine, and diazepam were found to have an additive antidotal effect on OT tremor, using doses and pretreatment times that produced only partial antagonism when the antidotes were administered separately (Fig. 3).

Effect of diazepam and l-hyoscyamine on oxotremorine-induced hypothermia

The effect of diazepam and l-hyoscyamine on OT-induced hypothermia is demonstrated in Table 1. l-Hyoscyamine (1 mg/kg, i.v.) administered 2 hr before the injection of OT (0.5 mg/kg, i.v.) partially blocked the hypothermia. Diazepam (1 mg/kg, i.v.), on the other hand, when injected 30 sec before OT accentuated the hypothermia but did not influence the antidotal effect achieved by l-hyoscyamine when the two antidotes were combined. Diazepam itself caused a maximal decrease in body temperature of about 2°C.

The above experiments indicate a selectivity in effect of diazepam on OT-induced tremor and hypothermia. Numerous investigations have suggested a role for hypothalamus in thermoregulation. Marks et al. (15) found no correlation between tolerance to OT-induced hypothermia and reduction in hypothalamic muscarinic receptor number measured in vitro with [³H]quinuclidinyl benzilate (QNB) binding technique, but suggest other mechanisms to be involved too. This is supported by the selectivity in the effect of diazepam found by us. The effect of diazepam on peripheral symptoms induced by OT was also selective. Diazepam pre-

vented diarrhea but had no effect on the salivation induced by OT. These effects were not quantitated but observed subjectively.

Muscarinic receptor density in vivo

The blocking effect of diazepam on OT-induced tremor raises questions as to whether diazepam itself affects muscarinic receptor binding. Since diazepam acts by potentiating the effect of GABA on the nerve terminals, resulting in hyperpolarization of the nerve membrane (16), this may induce a conformational change of the receptor.

Studies of muscarinic receptor concentration and comparative binding assays of agonists and antagonists are mostly done in vitro by incubation and measurement of binding to tissue homogenates of the radiolabelled potent antagonists [^3H]scopolamine or [^3H]QNB as described by Yamamura and Snyder (7). We have developed a technique that allows studies of the muscarinic receptor concentration to be performed in vivo under physiological conditions. The method is based on measurement of specific binding to muscarinic receptors of l-hyoscyamine, the "active" antipode of atropine. Fig. 4 demonstrated the concentration-time curve in cortex and cerebellum for l- and d-hyoscyamine injected i.v. in equal doses (1 mg/kg). d-Hyoscyamine disappeared rapidly from both cortex and cerebellum and its concentration was close to the detection limit (5 ng/g) within 2 hr. l-Hyoscyamine could be measured in cortex for 18 hr after injection. In cerebellum, however, known to be almost devoid of muscarinic receptors, the concentration of l-hyoscyamine declined rapidly. These results demonstrate that the pharmacologically "active" antipode binds much weaker to structures devoid of muscarinic receptors and that it differs from the "inactive" antipode d-hyoscyamine by binding more selectively to muscarinic receptors in cortex. The data show that l-hyoscyamine can be used for the study of muscarinic receptor binding in vivo in various regions of the mouse brain.

After administration of 1 mg/kg of l-hyoscyamine, its concentration in cortex, hippocampus, and striatum was the same after 18 hr (about 30 ng/g). At a dose of 4 mg/kg of l-hyoscyamine, its concentration after 6 hr tended to be somewhat higher in striatum compared to cortex and hippocampus (Fig. 5). In cerebellum, however, it was much lower. The concentrations of l-hyoscyamine 18 hr after administration were about the same as after 6 hr. This lasting concentration of about 60 ng l-hyoscyamine/g brain tissue was reached even when 2 mg/kg of l-hyoscyamine was administered (Fig. 6). One may therefore conclude that this concentration (60 ng/g) corresponds to the receptor concentration and that an excess of the drug is cleared more rapidly and within about 4 hr from unspecific binding sites. This concentration corresponds to a binding capacity in cortex of about 1400 pmol/g protein. Applying the *in vitro* binding technique with [³H]QNB, Nordberg and Larsson (17) determined the maximum binding capacity to 1200 pmol/g protein in mouse brain cortex.

The physiological significance of a lasting concentration of l-hyoscyamine reached after administration of 1 mg/kg i.v. was tested on its blocking effect on OT-induced tremor. Tremor is elicited by the muscarinic effects of OT and can be blocked by atropine but not by methylatropine, indicating the central nervous system origin of the effect. Mice given OT (0.1 mg/kg, i.v.) experienced pronounced tremor (Fig. 7). This tremor could be instantaneously blocked by injection of l-hyoscyamine (1 mg/kg, i.v.). Also, when mice had been pretreated for 2 hr with the same dose of l-hyoscyamine, the tremor-inducing effect of OT (0.1 mg/kg, i.v.) was blocked, which demonstrates the pharmacological effect of specifically bound low concentrations of l-hyoscyamine.

Effect of diazepam on muscarinic receptor binding

The influence of diazepam on muscarinic receptor density *in vivo* was studied by measuring the specific binding of l-hyoscyamine as described above (Table 2). l-Hyoscyamine was injected i.v. in a dose of 2 mg

(base)/kg, and its concentration in brain (half brain except cerebellum) was measured 2 hr after administration. In mice, when diazepam was injected (1 mg/kg, i.v.) 2 min before the administration of l-hyoscyamine, specific binding of l-hyoscyamine was markedly decreased. When the same dose of diazepam was injected 2 min after the dose of l-hyoscyamine, specific binding was still reduced but to a considerably less degree. Injection of diazepam much later after l-hyoscyamine, i.e. 2 min before the animals were killed reduced the binding of l-hyoscyamine to the same extent as when administered 2 min after l-hyoscyamine. These results suggest that when the receptor is already occupied by l-hyoscyamine, the conditions for a conformational change by diazepam might have been reduced, resulting in a lesser effect by diazepam on the specific binding of l-hyoscyamine to the receptors. In all three cases, tremor was counteracted by diazepam.

Effect of diazepam on the acetylcholine-synthesizing system of mouse brain

Pretreatment with diazepam (2 mg/kg, i.p.) for 20 min significantly increased the endogenous levels of ACh and Ch. At the same time it decreased the amount of [$^2\text{H}_6$]Ch reaching the brain and, as a consequence, decreased the concentration of [$^2\text{H}_6$]ACh (Table 3). These findings are partly in contrast to findings by Consolo et al. (3), who reported elevated levels of endogenous ACh in striatum and hippocampus of diazepam-treated rats but found no effect on the endogenous Ch levels. This might be due to the technique used for killing the animals (liquid nitrogen). We used focused microwave irradiation, which rapidly inactivates enzymes and prevents postmortem changes. A postmortem increase taking place before the analysis of Ch might have masked the effect of diazepam in their study.

Diazepam slightly decreased the turnover rate of ACh. The specific activity-time curves of deuterium-labelled ACh and Ch in controls and

diazepam-treated animals were consistent with a lowered turnover rate of ACh (Fig. 8, Table 4).

Diazepam increased the endogenous brain Ch level by 50% while the [$^2\text{H}_6$]Ch concentration was only about half of that of the controls. One possible explanation for these findings is that diazepam affects the Ch transport across the blood-brain barrier, either via a specific effect on Ch or via a general membrane effect, which would result in lower concentrations of [$^2\text{H}_6$]Ch reaching the brain. This hypothesis is supported by *in vitro* studies of Metlas et al. (6), in which a decreased uptake of [^3H]Ch into synaptosomes of diazepam-treated rats was demonstrated. Dross and Kewitz (18) have previously demonstrated a net outflow of Ch from the brain of rats. A decreased capacity of the Ch transport would thus lead to an accumulation of endogenous Ch in the brain and a smaller amount of exogenous Ch reaching it. This hypothesis is supported by experiments in which levels of endogenous and $^2\text{H}_6$ -labelled Ch were analyzed in whole blood following an i.v. injection of [$^2\text{H}_6$]Ch (Table 5). [$^2\text{H}_6$]Ch was found to be eliminated faster initially in animals treated with diazepam. Also, the increased level of endogenous Ch in blood, induced by the [$^2\text{H}_6$]Ch injection, returned more rapidly to normal. This indicates that in mice treated with diazepam, Ch in blood is eliminated faster due to a diminished supply of Ch from the brain. Diazepam itself did not influence the blood Ch level. The concentration of Ch in blood of mice treated with diazepam and saline, but not deuterium-labelled Ch i.v., was 24.0 ± 1.6 (6) and 24.2 ± 2.5 (5) nmol/ml, respectively.

These results support the theory that diazepam inhibits the uptake and elimination of brain Ch, resulting in elevated levels of endogenous Ch in the brain and a smaller amount of [$^2\text{H}_6$]Ch reaching it. To elucidate whether this effect of diazepam is a general effect or specific for Ch, we studied the effect of diazepam on the distribution of a cholinergically inactive compound, cotinine, to the brain of mice. Cotinine, being an uncharged tertiary amine at physiological pH, will pass through the

blood-brain barrier by passive diffusion in contrast to the charged quaternary amine Ch. The concentration-time curves are depicted in Fig. 9. No significant difference in the level of cotinine could be noticed in animals pretreated with diazepam compared to control animals. Thus, diazepam does not influence the distribution and elimination of an uncharged tertiary amine such as cotinine to the brain.

In further studies on the influence of diazepam on uptake and elimination of Ch in mouse brain, diazepam (1 mg/kg, i.v.) was administered either 1 min before or 1 min after the injection of [$^2\text{H}_6$]Ch (Fig. 10, Table 6). This design is an attempt to detect any differences in the effect of diazepam on uptake and elimination mechanisms of Ch, respectively. The concentration of endogenous and [$^2\text{H}_6$]Ch in brain was followed for 20 min. The levels of endogenous Ch increase very rapidly. Already, at the shortest time point studied (2.5 min), the concentration of endogenous Ch reached a level where it remained during the study, i.e., for at least 20 min. Injection of diazepam 1 min before [$^2\text{H}_6$]Ch demonstrates that the effect of diazepam on the uptake mechanism is immediate and is not mediated by a secondary effect of the increased level of endogenous Ch. Thus, the 2.5-min concentration is reduced in this experiment. By injecting [$^2\text{H}_6$]Ch 1 min before diazepam, the drug's effect on its elimination can be studied. After 2.5 min the concentration of [$^2\text{H}_6$]Ch is about the same as in control animals. When considering the elevated endogenous Ch by using specific activity curves of [$^2\text{H}_6$]Ch, as demonstrated in Fig. 11, the elimination seems to be initially slower in diazepam-treated animals. From 10 min and thereafter, the shapes of the curves are the same, demonstrating an unaffected rate of elimination of Ch from brain by diazepam. The shapes of the specific activity time curves of [$^2\text{H}_6$]ACh are consistent with a decreased turnover rate of ACh after diazepam administration, i.e., with a more flattened profile and a later appearing peak concentration. This implies a decrease of both the synthesis rate and release of ACh. The latter is confirmed by the results obtained when diazepam is given after [$^2\text{H}_6$]Ch in which the lowered rate of release can be detected.

CONCLUSIONS

It can be concluded that diazepam affects the cholinergic system in the brain of mice by increasing the level of ACh slightly and by lowering the turnover of ACh. The decrease of ACh turnover, consistent with a decrease in neuronal excitability (16), might be important for the antidotal effect obtained with diazepam in cases of organophosphate intoxication. The conventional antidote atropine blocks the cholinergic transmission postsynaptically but has been shown to increase TR_{ACh} (19). The combination of atropine and diazepam should therefore be more potent.

By measuring the physiologically "active" antipode l-hyoscyamine in mouse brain, it is possible to study the size of the muscarinic receptor pool and how this pool is influenced by treatment with cholinergically active compounds.

One possible mechanism responsible for the profound influence of diazepam on the effect of cholinergic stimulators, e.g., cholinesterase inhibitors, could be the exertion of a modulating effect on the binding properties of muscarinic receptors in the CNS. The blocking effect of diazepam on cholinergic stimulation by OT is in accord with its known stimulation of the GABAergic system, resulting in hyperpolarization of the nerve membrane and decreased nerve transmission.

The effect of diazepam on the Ch uptake system can be interpreted as a presynaptic event, while the effect on the receptor-binding properties can be both pre- and postsynaptic. Presynaptic receptor modification by diazepam may lead to the effects on the dynamics of Ch and ACh and thus be primary to these.

Thus, the effect of diazepam in the prophylaxis and treatment of organophosphate intoxications and the blocking properties of diazepam on central cholinergic symptoms, e.g., tremor induced by OT, are consis-

tent with the observed effects of diazepam on the dynamics of ACh and its suggested muscarinic receptor-modifying properties.

TABLE 1

Influence of diazepam and l-hyoscyamine on oxotremorine-induced hypothermia

Pretreatment	Decrease in body temperature (°C)
	1 hr after the OT administration Mean±SD (N)
OT (0.5 mg/kg, i.v.)	10.8±1.4(7)
Diazepam (1 mg/kg, i.v.) administered 30 sec be- fore the injection of OT (0.5 mg/kg, i.v.)	12.8±1.4(7)**
l-Hyoscyamine (1 mg/kg, i.v.) administered 2 hr before the injection of OT (0.5 mg/kg, i.v.)	8.9±0.4(6)**
l-Hyoscyamine (1 mg/kg, i.v.) and diazepam (1 mg/kg, i.v.) administered 2 hr and 30 sec, respectively, before the injection of OT (0.5 mg/kg, i.v.)	8.9±0.8(6)**

The decrease of body temperature (°C) is expressed as mean±SD with the number of animals within parentheses.

**2P<0.01, two-tailed Student's t-test of antidote plus OT treatment means in comparison with OT treatment mean. [From Nordgren et al. (20)]

TABLE 2

Influence of diazepam on specific binding of l-hyoscyamine (2 mg/kg, i.v.)

The concentration of l-hyoscyamine was measured 2 hr after administration, at which time it is supposed to reflect muscarinic receptor density.

Pretreatment	l-Hyoscyamine (ng/g) 2 hr after administration Mean±SD (N)
l-Hyoscyamine (2 mg/kg, i.v.)	57.7±5.7 (10)
Diazepam (1 mg/kg, i.v.) 2 min before the injection of l- hyoscyamine (2 mg/kg, i.v.)	34.3±8.1 (8)***
l-Hyoscyamine (2 mg/kg, i.v.) 2 min later followed by diazepam (1 mg/kg, i.v.)	50.3±5.7 (6)*
l-Hyoscyamine (2 mg/kg, i.v.) The mice received diazepam (1 mg/kg, i.v.) 2 min before they were sacrificed	50.8±3.0 (6)**

The data are mean±SD with the number of animals within parentheses.

*2P<0.05, **2P<0.01, ***2P<0.001, two-tailed Student's t-test of diazepam treatment means in comparison with the control mean. [From Nordgren et al. (20)]

TABLE 3

Effect of diazepam i.p. on endogenous and $^2\text{H}_6$ -substituted ACh and Ch in mouse brain

Concentration (nmol/g) of endogenous and $^2\text{H}_6$ -substituted ACh and Ch in whole brain of mice injected i.v. with 20 $\mu\text{mol/kg}$ [$^2\text{H}_6$]Ch and sacrificed 0.25 and 0.75 min later. The mice were pretreated with saline or diazepam (2 mg/kg, i.p.) 20 min prior to the [$^2\text{H}_6$]Ch injection. The data are mean $\pm$ SD with the number of animals within parentheses.

		Pretreatment	
		Saline	Diazepam
Time (min)			
ACh	0.25	19.0 $\pm$ 2.0 (16)	21.2 $\pm$ 2.4 (17)**
	0.75	20.1 $\pm$ 1.5 (18)	20.6 $\pm$ 1.7 (17)n.s.
Ch	0.25	32.6 $\pm$ 10.5 (16)	45.1 $\pm$ 8.0 (17)***
	0.75	30.1 $\pm$ 7.4 (18)	47.1 $\pm$ 9.6 (17)***
[$^2\text{H}_6$]ACh	0.25	0.247 $\pm$ 0.083 (16)	0.115 $\pm$ 0.024 (17)***
	0.75	0.656 $\pm$ 0.172 (18)	0.249 $\pm$ 0.052 (17)***
[$^2\text{H}_6$]Ch	0.25	6.29 $\pm$ 2.13 (16)	3.26 $\pm$ 0.82 (17)***
	0.75	3.51 $\pm$ 1.08 (18)	2.80 $\pm$ 0.74 (17)***

Two-tailed Student's t-test of treatment means in comparison with saline means.

2P<0.01, *2P<0.001, n.s. = not significant. [From Lundgren et al. (21)]

TABLE 4

Effect of diazepam i.p. on specific activity of $^2\text{H}_6$ -substituted ACh and Ch, fractional rate constant and turnover rate of ACh in mouse brain

Specific activity (S) (mole ratio, mean $\pm$ SD with the number of animals within parentheses) of $^2\text{H}_6$ -substituted ACh and Ch, fractional rate constant (K_a), and turnover rate of ACh (TR_{ACh}) in whole brain of mice. Experimental conditions as for Table 3. K_a (min^{-1}) was calculated by the Zilversmit (11) equation from S_{ACh} and S_{Ch} at 0.25 and 0.75 min. TR_{ACh} was obtained by multiplying K_a with the endogenous concentration of ACh.

	Time (min)	Pretreatment	
		Saline	Diazepam
S_{ACh}	0.25	0.0130 $\pm$ 0.0044 (16)	0.0054 $\pm$ 0.0013 (17)***
	0.75	0.0318 $\pm$ 0.0091 (18)	0.0120 $\pm$ 0.0025 (17)***
S_{Ch}	0.25	0.169 $\pm$ 0.053 (16)	0.0687 $\pm$ 0.0189 (17)***
	0.75	0.107 $\pm$ 0.032 (18)	0.0639 $\pm$ 0.0291 (17)***
$K_a \text{ min}^{-1}$		0.33	0.23
TR_{ACh} nmol/g x min		6.38	4.76

Two-tailed Student's t-test of treatment means in comparison with saline means.

***2P<0.001. [From Lundgren et al. (21)]

TABLE 5

Effect of diazepam i.p. on specific activity and concentrations of endogenous and $^2\text{H}_6$ -substituted Ch in whole blood of mice

Specific activity (mole ratio, S) and concentration (nmol/ml) of endogenous and $^2\text{H}_6$ -substituted Ch in whole blood of mice injected i.v. with 20 $\mu\text{mol/kg}$ [$^2\text{H}_6$]Ch and sacrificed 0.25 and 0.75 min later. The mice were pretreated with saline or diazepam (2 mg/kg, i.p.) 20 min prior to the [$^2\text{H}_6$]Ch injection. The concentrations of endogenous Ch in the blood of mice before the injection of [$^2\text{H}_6$]Ch in saline and diazepam-treated animals were 24.2 ± 2.5 (5) and 24.0 ± 1.6 (6) nmol/ml, respectively. The data are mean $\pm$ SD with the number of animals within parentheses.

		Pretreatment	
		Saline	Diazepam
Ch	0.25	37.2 ± 3.1 (10)	30.5 ± 4.2 (10)***
	0.75	34.2 ± 3.0 (10)	32.0 ± 4.7 (10)n.s.
[$^2\text{H}_6$]Ch	0.25	55.2 ± 13.8 (10)	39.4 ± 7.0 (10)***
	0.75	13.9 ± 4.6 (10)	11.9 ± 1.8 (10)n.s.
S_{Ch}	0.25	0.588 ± 0.064 (10)	0.556 ± 0.056 (10)n.s.
	0.75	0.284 ± 0.067 (10)	0.271 ± 0.039 (10)n.s.

Two-tailed Student's t-test of treatment means in comparison with saline means.

*** $2P < 0.001$, n.s. = not significant. [From Lundgren et al. (21)]

TABLE 6

Effect of diazepam i.v. on specific activity and concentration of endogenous and $^2\text{H}_6$ -substituted ACh and Ch in mouse brain

Specific activity (mole ratio, S) and concentration (nmol/g) of endogenous and $^2\text{H}_6$ -substituted ACh and Ch in whole brain of mice injected i.v. with 20 $\mu\text{mol/kg}$ [$^2\text{H}_6$]Ch and sacrificed 2.5-20 min later. The mice were administered diazepam (1 mg/kg, i.v.) 1 min prior to or 1 min after the [$^2\text{H}_6$]Ch injection. The data are mean $\pm$ SD with the number of animals within parentheses.

	Time (min)	Treatment		
		Saline	Diazepam injection 1 min prior to [$^2\text{H}_6$]Ch	Diazepam injection 1 min after [$^2\text{H}_6$]Ch
ACh	2.5	18.2 $\pm$ 2.8(12)	21.2 $\pm$ 2.0(9)*	20.5 $\pm$ 2.6(12)*
	5	19.0 $\pm$ 1.8(16)	22.9 $\pm$ 1.9(8)***	20.9 $\pm$ 3.3(10)n.s.
	10	18.9 $\pm$ 2.9(16)	21.1 $\pm$ 1.4(12)*	22.1 $\pm$ 3.0(9)*
	20	18.4 $\pm$ 1.6(9)	20.8 $\pm$ 2.5(10)*	20.7 $\pm$ 3.3(10)n.s.
Ch	2.5	35.7 $\pm$ 5.1(12)	54.1 $\pm$ 10.8(9)***	43.6 $\pm$ 9.5(12)*
	5	38.1 $\pm$ 7.4(16)	48.9 $\pm$ 9.2(8)**	39.8 $\pm$ 4.0(10)n.s.
	10	36.5 $\pm$ 6.3(16)	45.9 $\pm$ 5.9(12)***	43.5 $\pm$ 6.6(9)*
	20	31.0 $\pm$ 2.8(9)	54.1 $\pm$ 12.4(10)***	55.2 $\pm$ 13.0(10)***
[$^2\text{H}_6$]ACh	2.5	0.866 $\pm$ 0.134(12)	0.676 $\pm$ 0.104(9)**	0.935 $\pm$ 0.127(12)n.s.
	5	0.740 $\pm$ 0.124(16)	0.741 $\pm$ 0.201(8)n.s.	1.008 $\pm$ 0.156(10)***
	10	0.623 $\pm$ 0.151(16)	0.634 $\pm$ 0.208(12)n.s.	0.936 $\pm$ 0.205(9)***
	20	0.379 $\pm$ 0.110(9)	0.508 $\pm$ 0.129(10)*	0.647 $\pm$ 0.175(10)**
[$^2\text{H}_6$]Ch	2.5	2.11 $\pm$ 0.32(12)	1.56 $\pm$ 0.38(9)**	2.06 $\pm$ 0.45(12)n.s.
	5	1.29 $\pm$ 0.52(16)	1.35 $\pm$ 0.36(8)n.s.	1.58 $\pm$ 0.36(10)n.s.
	10	0.873 $\pm$ 0.194(16)	0.837 $\pm$ 0.221(12)n.s.	1.03 $\pm$ 0.19(9)n.s.
	20	0.418 $\pm$ 0.130(9)	0.613 $\pm$ 0.239(10)*	0.670 $\pm$ 0.115(10)***
S _{ACh}	2.5	0.0463 $\pm$ 0.0078(12)	0.0310 $\pm$ 0.0037(9)***	0.0442 $\pm$ 0.0052(12)n.s.
	5	0.0376 $\pm$ 0.0053(16)	0.0311 $\pm$ 0.0071(8)*	0.0461 $\pm$ 0.0034(10)***
	10	0.0322 $\pm$ 0.0070(16)	0.0291 $\pm$ 0.0090(12)n.s.	0.0404 $\pm$ 0.0059(9)**
	20	0.0200 $\pm$ 0.0046(9)	0.0245 $\pm$ 0.0088(10)n.s.	0.0302 $\pm$ 0.0047(10)***
S _{Ch}	2.5	0.0564 $\pm$ 0.0099(12)	0.0289 $\pm$ 0.0090(9)***	0.0473 $\pm$ 0.0146(12)n.s.
	5	0.0328 $\pm$ 0.0107(16)	0.0265 $\pm$ 0.0081(9)n.s.	0.0381 $\pm$ 0.0072(10)n.s.
	10	0.0238 $\pm$ 0.0057(16)	0.0179 $\pm$ 0.0041(12)**	0.0227 $\pm$ 0.0060(9)n.s.
	20	0.0132 $\pm$ 0.0038(9)	0.0111 $\pm$ 0.0029(10)n.s.	0.0123 $\pm$ 0.0023(10)n.s.

Two-tailed Student's t-test of treatment means in comparison with saline means.

*2P<0.05, **2P<0.01, ***2P<0.001, n.s. = not significant.

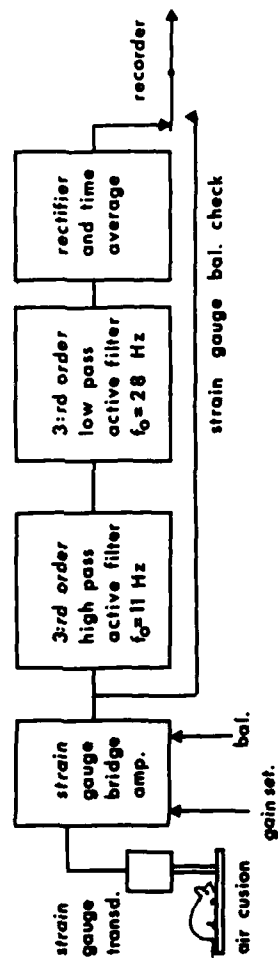


Figure 1. Block diagram of equipment for recording of tremor. [From Palmér et al. (22)]

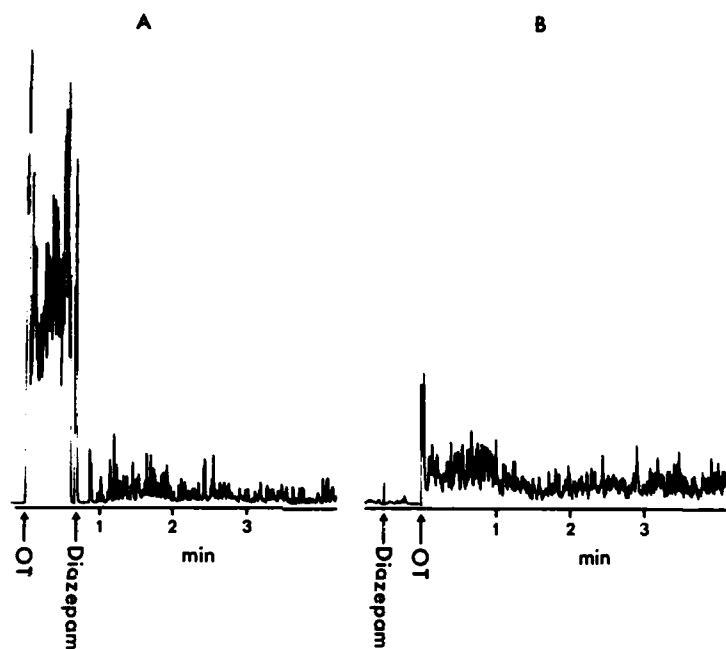


Figure 2. Effect of diazepam on tremor induced by oxotremorine. A, OT (0.5 mg/kg, i.v.) followed by diazepam (1 mg/kg, i.v.); B, diazepam (1 mg/kg, i.v.) administered 30 sec before the injection of OT (0.5 mg/kg, i.v.). [From Nordgren et al. (20)].

Two-tailed Student's *t*-test of diazepam treatment means (2 mice) in comparison to the mean of the tremor induced by OT: A, $2P < 0.01$; B, $2P < 0.01$.

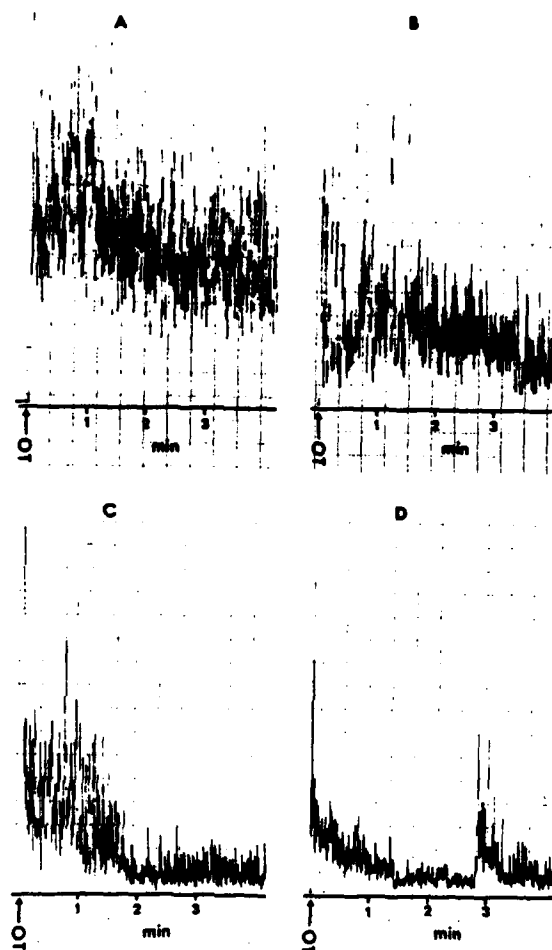


Figure 3. Effect of diazepam and l-hyoscyamine on tremor induced by oxotremorine. A, OT (0.5 mg/kg, i.v.); B, l-hyoscyamine (1 mg/kg, i.v.) administered 2 hr before the injection of OT (0.5 mg/kg, i.v.); C, diazepam (2 mg/kg, i.p.) administered 20 min before the injection of OT (0.5 mg/kg, i.v.); D, l-hyoscyamine (1 mg/kg, i.v.) and diazepam (2 mg/kg, i.p.) administered 2 hr and 20 min, respectively, before the injection of OT (0.5 mg/kg, i.v.). [From Nordgren et al. (20)].

Two-tailed Student's t-test of diazepam and/or l-hyoscyamine treatment means (4-7 mice) in comparison to the mean of the tremor induced by OT (A): B, $2P < 0.05$; C, $2P < 0.10$; D, $2P < 0.001$.

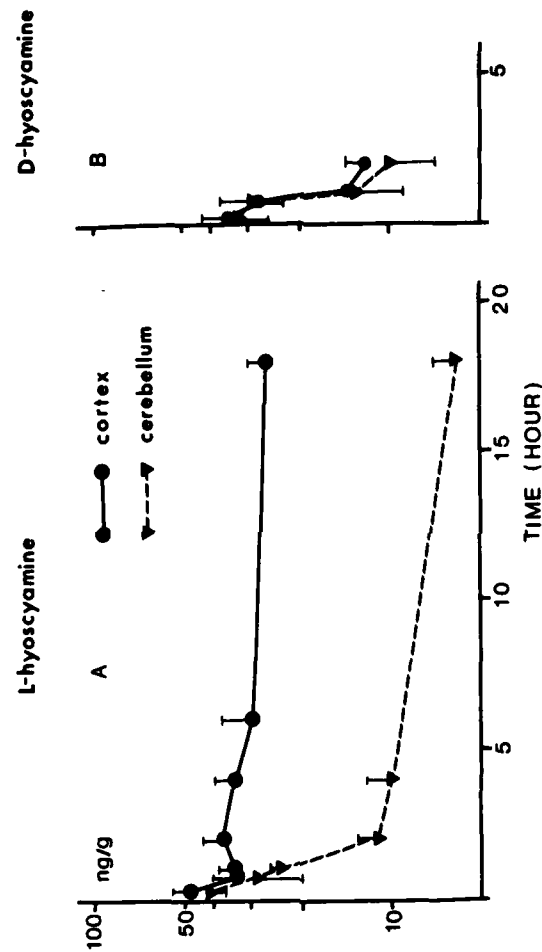


Figure 4. Elimination of d- and l-hyoscyamine in mouse brain. Concentration-time curves of l- and d-hyoscyamine in cortex and cerebellum following i.v. separate injection of the two drugs at a dose of 1 mg/kg. The concentrations are means $\pm$ SD of four to seven mice. [From Palmér et al. (22)]

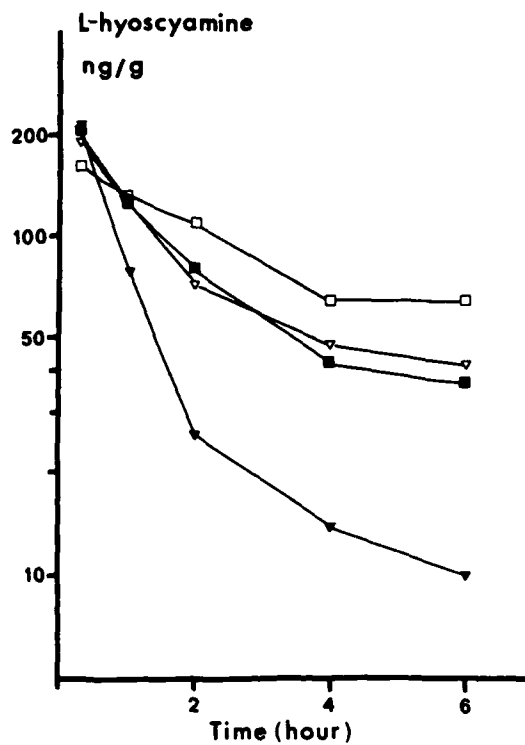


Figure 5. Concentration of l-hyoscyamine in different parts of the brain. The mice were administered 4 mg/kg i.v. Each point represents mean values obtained from two to eight mice.
□, Striatum, ▽, Cortex, ■, Hippocampus, ▼, Cerebellum. [From Palmér et al. (23)]

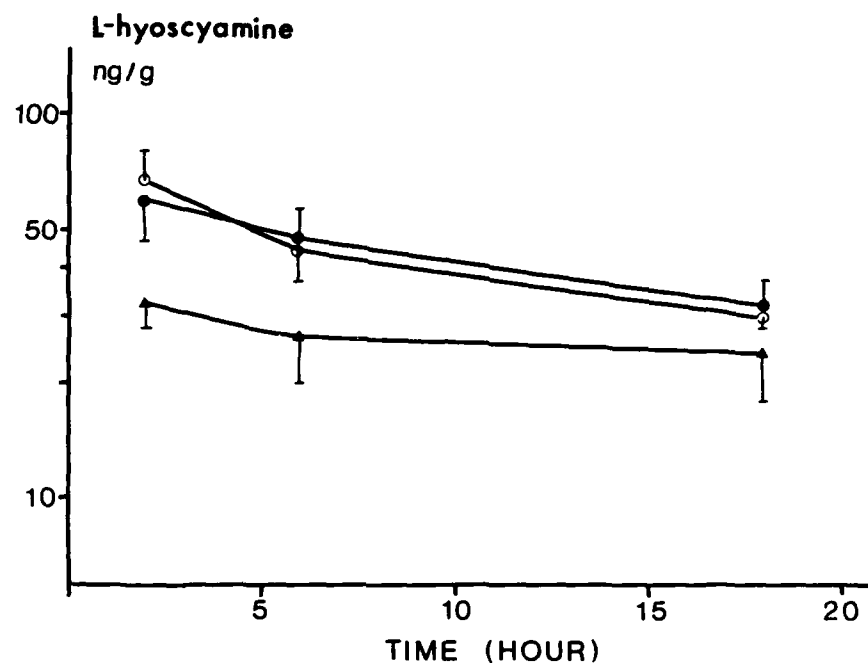


Figure 6. Concentrations - time curves of l-hyoscyamine in cortex following injection of the drug at doses of 1 (▲), 2 (●), and 4 mg/kg (○), respectively. The concentrations are means $\pm$ S.D. of 5-6 mice. Student's impaired two-tailed t-test of differences between means 2-4 mg/kg (2, 6 and 18 hr): $P > 0.1$. 1-2 mg/kg (2 and 6 hr): $P < 0.001$. 1-2 mg/kg (18 hr): $P < 0.05$. [From Palmér et al. (22)]

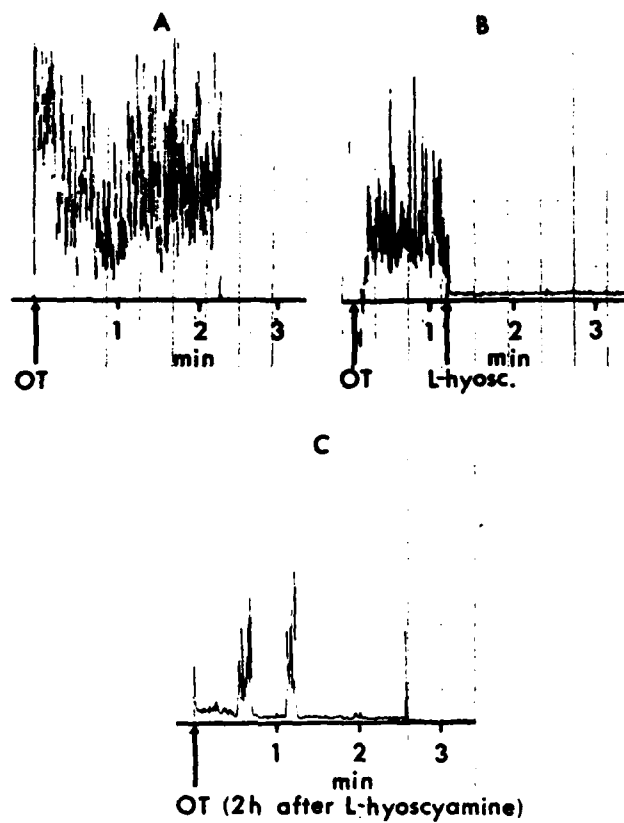


Figure 7. Effect of specifically bound l-hyoscyamine on oxotremorine induced tremor. A, OT (0.1 mg/kg, i.v.); B, OT (0.1 mg/kg, i.v.) followed 1 min later by l-hyoscyamine (1 mg/kg, i.v.); C, OT (0.1 mg/kg, i.v.) 2 hr after l-hyoscyamine (1 mg/kg, i.v.). [From Palmér et al. (22)]

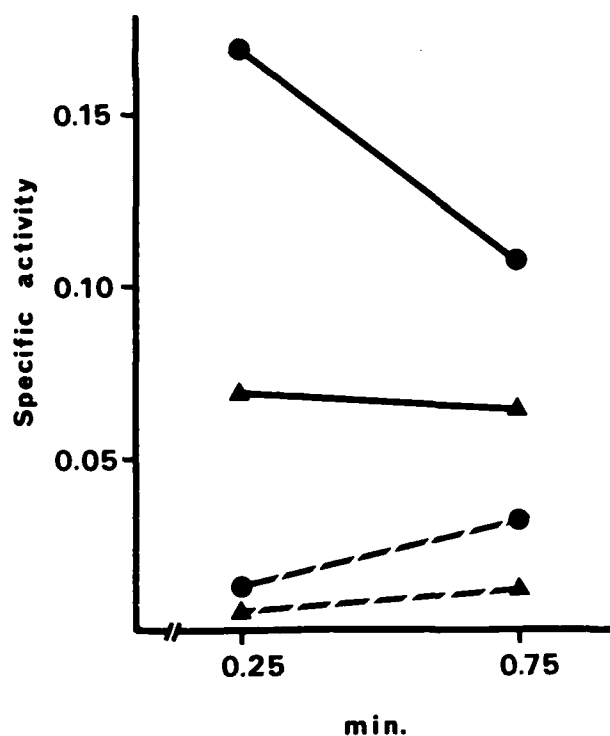


Figure 8. Specific activity-time curves of ACh (---) and Ch (—) in whole brain of mice after pretreatment with diazepam. The mice were injected i.v. with 20 $\mu\text{mol/kg}$ [$^2\text{H}_6$]Ch 20 min after pretreatment i.p. with saline (●) or diazepam (▲), 2 mg/kg. [From Lundgren et al. (21)]

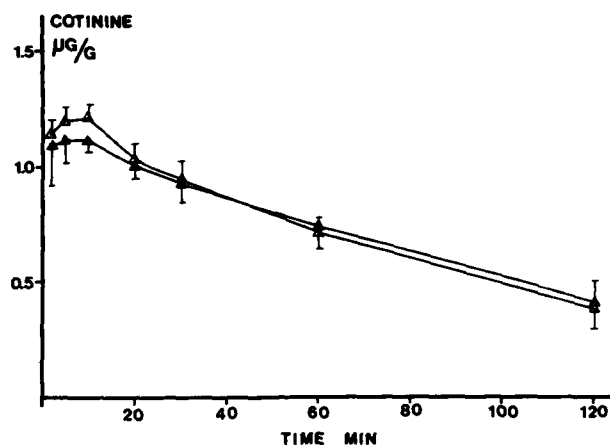


Figure 9. Effect of diazepam on uptake and elimination of cotinine in mouse brain. Concentration-time curves of cotinine in whole brain of mice injected i.v. with cotinine, 2 mg/kg. The mice were untreated (Δ) or pretreated with diazepam ($\blacktriangle$) (2 mg/kg, i.p.) 20 min prior to the cotinine injection. Each point represents mean values obtained from five mice (at 120 min, three mice were used). Error bars = SD.

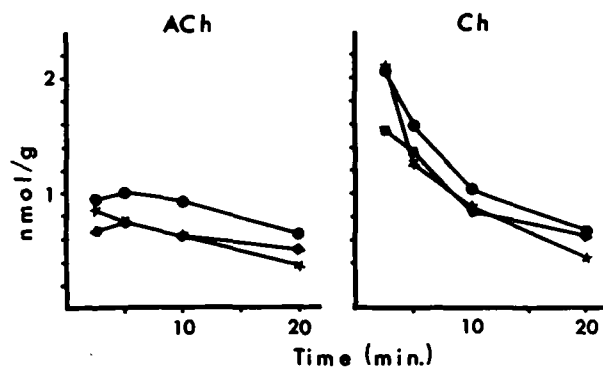


Figure 10. Effect of diazepam on concentration of $[^2\text{H}_6]\text{ACh}$ and $[^2\text{H}_6]\text{Ch}$ in whole brain of mice. The mice were injected i.v. with $[^2\text{H}_6]\text{Ch}$ (20 $\mu\text{mol/kg}$).

- ★ , No diazepam administered;
- ◆ , diazepam (1 mg/kg, i.v.) administered 1 min prior to the injection of $[^2\text{H}_6]\text{Ch}$;
- , diazepam (1 mg/kg, i.v.) administered 1 min after the injection of $[^2\text{H}_6]\text{Ch}$.

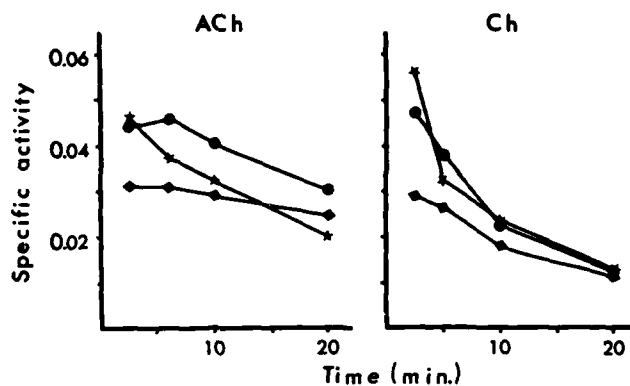


Figure 11. Effect of diazepam i.v. on specific activity - time curves of deuterium labelled ACh and Ch in whole brain of mice. The mice were injected i.v. with [²H₆]Ch (20 μ mol/kg).

- ★, No diazepam administered
- ◆, diazepam (1 mg/kg, i.v.) administered 1 min prior to the injection of [²H₆]Ch
- , diazepam (1 mg/kg, i.v.) administered 1 min after the injection of [²H₆]Ch.

REFERENCES

1. Boscovic, B.: The treatment of soman poisoning and its perspectives. *Fund. Appl. Toxicol.* 1981, 1, 203-213.
2. Krutak-Krol, H. and Domino, E.F.: Comparative effects of diazepam and midazolam on paraoxon toxicity in rats. *Toxicol. Appl. Pharmacol.* 1985, 81, 545-550.
3. Consolo, S., Garattini, S. and Ladinsky, H.: Action of the benzodiazepines on the cholinergic system. In: *Mechanism of Action of Benzodiazepines*. E.Costa and P. Greengard (Eds.). Raven Press, New York, 1975, pp. 63-80.
4. Kolasa, K., Consolo, S., Forloni, G., Garattini, S. and Ladinsky, H.: Blockade of the diazepam-induced increase in rat striatal acetylcholine content by the specific benzodiazepine antagonists ethyl- β -carboline-3-carboxylate and Ro15-1788. *Brain Res.* 1985, 336, 342-345.
5. Phillis, J.W., Siemens, R.K. and Wu, P.H.: Effect of diazepam on adenosine and acetylcholine release from rat cerebral cortex: Further evidence for a purinergic mechanism in action of diazepam. *Br. J. Pharmacol.* 1980, 70, 314-348.
6. Metlas, R., Horvat, A., Nikezic, G., Cetkovic, S. and Boscovic, B.: Acetylcholine synthesis and release by brain cortex synaptosomes of rats treated with diazepam. *Jugosl. Physiol. Pharmacol. Acta*, 1984, 20, 213-218.
7. Yamamura, H.J. and Snyder, S.H.: Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. USA*, 1974, 71, 1725-1729.

8. Karlén, B., Lundgren, G., Nordgren, I. and Holmstedt, B.: Ion pair extraction and gas phase analysis of acetylcholine and choline. In: Choline and Acetylcholine. Handbook of Chemical Assay Methods. I. Hanin (Ed.). Raven Press, New York, 1974, pp. 163-179.
9. Palmér, L., Edgar, J., Lundgren, G., Karlén, B. and Hermanson, J.: Atropine in mouse brain and plasma quantified by mass fragmentography. *Acta Pharmacol. Toxicol.* 1981, 49, 72-76.
10. Karlén, B. and Telc, A.: Synthesis of tritiated oxotremorine, labelled in the pyrrolidine ring. *Acta Pharm. Suec.* 1966, 3, 197-200.
11. Zilversmit, D.B.: The design and analysis of isotope experiments. *Am. J. Med.* 1960, 29, 832-848.
12. Karlén, B., Lundgren, G., Lundin, J. and Holmstedt, B.: On the turnover of acetylcholine in mouse brain: Influence of dose size of deuterium labelled choline given as precursor. *Biochem. Pharmacol.* 1982, 31(18), 2867-2872.
13. Nordgren, I., Lundgren, G., Puu, G., Karlén, B. and Holmstedt, B.: Distribution and elimination of the stereoisomers of soman and their effect on brain acetylcholine. *Fund. Appl. Toxicol.* 1985, 5, S252-S259.
14. Olsson, G.L., Bejersten, A., Feychting, H., Palmér, L. and Pettersson, B.-M.: Plasma concentrations of atropine after rectal administration. *Anaesthesia*, 1983, 38, 1179-1182.
15. Marks, M.J., Artman, L.D., Patinkin, D.M. and Collins, A.C.: Cholinergic adaptations to chronic oxotremorine infusion. *J. Pharmacol. Exp. Ther.* 1981, 218(2), 337-343.

16. Paul, S.M., Marangos, P. and Skolnik, P.: The benzodiazepine-GABA-chloride-ionophore receptor complex: Common site of minor tranquilizer action. *Biol. Psychiatry*, 1981, 16, 213-229.
17. Nordberg, A. and Larsson, C.: Studies of muscarinic and nicotinic binding sites in brain. *Acta Physiol. Scand.* 1980, Suppl. 479, 19-23.
18. Dross, K. and Kewitz, H.: Concentration and origin of choline in the rat brain. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 1972, 274, 91-106.
19. Karlén, B., Lundgren, G., Lundin, J. and Holmstedt, B.: Effect of physostigmine and atropine on acetylcholine turnover in mouse brain. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 1979, 308, 61-65.
20. Nordgren, I., Lundgren, G. and Karlén, B.: Effects of diazepam on muscarinic acetylcholine receptor binding in vivo and on oxotremorine-induced tremor and hypothermia in mice. *Pharmacology and Toxicology*, 1987, 60, 258-261.
21. Lundgren, G., Nordgren, I., Karlén, B. and Jacobsson, G.: Effects of diazepam on blood choline and acetylcholine turnover in brain of mice. *Pharmacology and Toxicology*, 1987, 60, 96-99.
22. Palmér, L., Lundgren, G. and Karlén, B.: A method using l-hyoscyamine for the study of muscarinic acetylcholine receptor binding in vivo. *Pharmacology and Toxicology*, 1987, 60, 54-57.
23. Palmér, L., Lundgren, G., Holmstedt, B. and Karlén, B.: Binding of l-hyoscyamine as a method to study muscarinic acetylcholine receptor density in vivo. In: *Cellular and Molecular Basis of Cholinergic Function*. Dowdall and Hawthorne (Eds.). Ellis Horwood Ltd., Chichester, England, and VCH, Weinheim, FRG, 1987, pp. 86-90.

ABBREVIATIONS

ACh, Acetylcholine

DPA, Dipicrylamine (2,4,6,2',4',6'-hexanitrodiphenylamine)

Ch, Choline

BSA, N,O-bis(trimethylsilyl)-acetamide

GC-MS, Gas chromatography-mass spectrometry

OT, Oxotremorine

S_{ACh} , Specific activity of [2H_6]ACh

S_{Ch} , Specific activity of [2H_6]Ch

TR_{ACh} , Acetylcholine turnover

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