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Anticonvulsant and Other Effects of Diazepam Grow With Time After a Single Treatment

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ANTELMAN, S. M., D. KOCAN, D. J. EDWARDS AND S. KNOPF. *Anticonvulsant and other effects of diazepam grow with time after a single treatment.* PHARMACOL BIOCHEM BEHAV **33**(1) 31–39, 1989.—The hypothesis was tested that some of the effects in rats of the prototypical benzodiazepine, diazepam, would grow (i.e., sensitize) with the passage of time after acute administration as we had previously observed following stimulants, antidepressants, neuroleptics and other compounds. Our principal findings indicate that: 1) A single pretreatment with 0.5 mg/kg of diazepam significantly enhances the anticonvulsant effect of this same dose administered again two weeks later. 2) One injection of 2.5 mg/kg of diazepam significantly sensitizes the catalepsy and ptosis observed following the administration of haloperidol two weeks but not two hours later. These data provide the first evidence for time-dependent sensitization after benzodiazepines and perhaps by implication, of GABA neurons. They may also suggest that acute stimulation of GABA neurons triggers the progressive development of a long-term, antidopaminergic influence. Finally, they raise the question of whether the progressive anxiolytic influence seen during the first week or so of benzodiazepine therapy depends on the passage of time rather than repeated drug treatment.

Time-dependent sensitization Diazepam Benzodiazepines Pentylenetetrazole Convulsions Stress Dopamine Neuroleptics

A fundamental and tacit assumption underlying the use of essentially all pharmacological agents both in the laboratory and the clinic is that any effect which either appears or is enhanced with repeated treatment depends on such treatment. An alternative possibility virtually never considered is that first exposure to a drug initiates a cellular process which then grows independently even after the inducing agent has left the system and manifests itself when the organism is later reexposed to that same or a similar agent (2). Yet, precisely such a time-dependent sensitization (TDS) process begins to develop in the immune system when an organism is first exposed to a foreign substance of sufficient size (an antigen) and is revealed if and when reexposure occurs some time later (16). Although the molecular weight of most drugs may not be sufficient to trigger an immune response (23), almost all represent foreign substances to the organism and to the extent that foreignness is the critical variable in TDS, they would be expected to induce this phenomenon.

Consistent with such a possibility, TDS has now been demonstrated after acute administration of a host of diverse agents, including the antidepressants, imipramine (14), amitriptyline (6), desmethylimipramine (27), chlorimipramine (36), phenelzine (4), and bupropion (1), the stimulant, amphetamine (5,41), the hallucinogenic, phencyclidine (unpublished observations) and the antipsychotics, haloperidol and fluphenazine hydrochloride (7). It has also been seen after a single exposure to the cholinergic agonist, oxotremorine (30), the dopamine agonist, apomorphine (34) and the benzodiazepine partial inverse agonist, FG7142 (47). The fact that TDS is inducible by such a diverse array of psychotropic agents lends credence to our hypothesis that it represents a general response of the nervous system to acute drug treatment. We further examined the generality of this phenomenon in the present studies by testing whether diazepam, the prototype of the benzodiazepines, is also capable of showing TDS.

METHOD

Animals

Two hundred and eighty-seven, naive, male, Sprague-Dawley rats from Zivic-Miller Farms, Allison Park, PA and weighing

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120–150 g at the time of purchase were used in these experiments. They were double-housed in wire-mesh cages to avoid the stress of isolation and maintained on a timed, temperature-controlled natural day/night cycle, with lights on at 0600 and off at 1800 hours. Experiments were begun after animals had a week to adjust to the laboratory and food and water were available ad lib. They were weighed daily to insure that growth was proceeding normally and to prevent the hyperreactivity which occurs when animals are left unhandled for long periods. Every effort is made to insure that nonexperimental stressors are held to a minimum in our laboratory. Access to the colony room (which is noncommunal) is strictly limited and care is carried out with very little noise or disturbance to the animals. A measure of our success in maintaining a low stress environment can be seen in that plasma corticosterone levels in our untreated animals have ranged from 1 to <3 μg/dl (3).

Anticonvulsant Effects of Diazepam and TDS

The first question asked was whether the well-known ability of diazepam to antagonize pentylenetetrazol (PTZ)-induced seizures (32) shows evidence of TDS, i.e., does the impact of a marginally effective dose grow with the passage of time. PTZ was administered IP at 40 mg/kg, a dose which preliminary testing determined induced clonic-tonic convulsions in approximately 90% of the animals. The dose of diazepam was 0.5 mg/kg, IP. Animals were grouped according to the following experimental conditions. A total of twenty-four control rats received either PTZ itself (N = 12), PTZ preceded by a single saline injection 14 days earlier (saline—14 days→PTZ; N = 6) or saline injections both 14 days and 1 hour prior to PTZ (saline—14 days→saline—1 hour→PTZ; N = 6). An additional 24 control animals received an injection of diazepam 1 hour before PTZ. These were divided into a no additional pretreatment group (diaz.—1 hour→PTZ; N = 12) and animals that also received a single injection of saline 14 (N = 6) or 28 (N = 6) days prior to diazepam (saline—14 or 28 days→diaz.—1 hour→PTZ). A third control condition consisted of 16 animals, which received a single injection of diazepam 14 (N = 10) or 28 (N = 6) days before PTZ (diaz.—14 or 28 days→PTZ). Experimental animals received two injections of diazepam prior to PTZ, spaced 14 (N = 10) or 28 (N = 10) days apart (diaz.—14 or 28 days→diaz.—1hour→PTZ).

Antistress Effects of Diazepam and TDS

It has been demonstrated that diazepam can prevent the effects of a stressor on dopamine (DA) metabolism in mesocortical and mesolimbic (18,29) brain regions. Even apart from its convulsant action, PTZ can be considered to be a stressor since it induces a pronounced (12–25-fold) increase in rat plasma corticosterone levels [(3) and unpublished observations], is thought to be a prototypical anxiogenic agent in rats (28) and to induce feelings of catastrophic anxiety in humans (42,43). We therefore used it to determine whether the antistress effects of diazepam on DA metabolism showed evidence of TDS. This experiment was done in those animals described in the last section which received two diazepam treatments spaced 28 days apart prior to PTZ (diaz.—28 days→diaz.—1 hr→PTZ). They were sacrificed by guillotine 10 minutes after PTZ, by which time all seizure activity was complete. Frontal cortices (FC) and accumbens nuclei were immediately dissected using an ice-cold "brain-block" according to the procedure of Heffner *et al.* (22) and stored at −50°C until assay.

DA and DOPAC were determined by an adaption of the procedures of Reinhard and Roth (38). A 0.5 ml aliquot of each supernatant solution (obtained from samples homogenized in 0.1 M $HClO_4$) was transferred to a 1.5 ml Beckman microfuge tube. After adding an appropriate amount (typically 60 ng) of the internal standard, DHBA (3,4-dihydroxybenzylamine), 200 μl of 1 M (pH 8.6) Tris buffer (containing 0.2 M EDTA and 3 mM sodium metabisulfite) and 50 mg of alumina were added, and the tubes were immediately mixed for 10 minutes to allow maximal adsorption of the catechols by the alumina. The alumina was washed with 1 ml of 0.1 M (pH 7.0) Tris buffer containing 10 mM EDTA and 1 mM sodium metabisulfite, and the catechols were eluted into 300 μl of 0.1 M $HClO_4$. Part of each eluate (50–100 μl) was injected into a model LC-153 Bio-Analytical Systems, Inc. (West Lafayette, IN) HPLC equipped with a 5 μm Biophase ODS reverse phase precolumn and column. The mobile phase, which consisted of 0.1 M KH_2PO_4, 0.1 mM EDTA, 1.5 mM sodium octylsufate, and 14% methanol (pH 3.2), was pumped at a flow rate of 1.5 ml/min (3000 psi). Catecholic compounds were detected by a TL-5 glassy carbon electrode maintained by an LC-3A controller at an oxidation potential of 0.9 V vs. an Ag/AgCl reference electrode.

Diazepam and TDS of Neuroleptic-Induced Catalepsy and Ptosis

By facilitating GABA transmission, benzodiazepines indirectly exert a complex modulatory influence on DA neurons (21) and in this way can alter behavioral and biochemical effects of neuroleptics. For example, at high (10 mg/kg) doses, diazepam has been shown to potentiate neuroleptic-induced catalepsy (37). Since we have previously found that a single pretreatment with a neuroleptic was sufficient to sensitize the cataleptic response to that same agent when it was administered for only the second time up to at least two months later (7), we inquired whether substituting diazepam for the initial treatment would also lead to TDS of neuroleptic-induced catalepsy. This was accomplished by administering diazepam (2.5 mg/kg) or saline two hours (N's = 8 and 6), 14 days (N's = 10 and 10) or 28 days (N's = 10 and 10) prior to 0.4 mg/kg, IP of haloperidol. A group receiving haloperidol without any preinjection was also run (N = 6). The degree of forepaw catalepsy on a 6.5-cm high platform was then determined in 2-minute tests conducted at intervals between 5 and 60 minutes after neuroleptic injection. Since ptosis is thought to reflect the sedative action of neuroleptics (35) and sedation is one of the principal effects of benzodiazepines, we also sought to determine whether diazepem could induce TDS of ptosis to later haloperidol treatment. Ptosis was scored according to the procedure used by Niemegeers (35) where 0 = eyes completely open, 2 = eyes half closed, 4 = eyes completely closed and 1 and 3 = intermediate scores. The time of catalepsy testing was counterbalanced across groups.

The same experiment was also repeated using only a 2-hour and 14-day interval between diazepam and haloperidol, with a subset of the 14-day interval animals receiving pretreatment with the benzodiazepine receptor antagonist, RO15-1788 or its vehicle (see the TDS and RO15-1788 section) (24). Assessment of the DOPAC response to haloperidol in the striatum, accumbens and FC was made at the conclusion of the experiment.

TDS and RO15-1788

Since TDS has now been demonstrated after a broad array of compounds (see Introduction) including antagonists (7) as well as agonists, we inquired whether RO15-1788 could also induce this phenomenon. Specifically, two injections of a low dose of RO15-1788 (1.5 mg/kg, IP) were administered 14 days apart and the ability noted of the second injection to antagonize the anticon-

TABLE 1

THE EFFECTS OF SPACED DIAZEPAM (0.5 mg/kg) TREATMENTS ON PTZ- (40 mg/kg) INDUCED CHANGES IN DOPAMINE AND DOPAC

	Nucleus Accumbens			Frontal Cortex		
	% of Control			% of Control		
Group	DA	DOPAC	DOPAC/DA	DA	DOPAC	DOPAC/DA
No Treatment	100 ± 4	100 ± 5	1.01 ± 0.09	100 ± 18	100 ± 20	1.00 ± 0.22
PTZ	142 ± 8*	100 ± 11	0.71 ± 0.07†	223 ± 57†	122 ± 12	0.55 ± 0.15
Diazepam—1 hr→PTZ	79 ± 10‡	100 ± 10	1.25 ± 0.08‡	94 ± 7‡	68 ± 10	0.72 ± 0.14
Saline—28 days→diazepam—1 hr→PTZ	129 ± 5§	100 ± 3	0.78 ± 0.04§	169 ± 25§	94 ± 14	0.56 ± 0.07
Diazepam—28 days→PTZ	123 ± 11	100 ± 10	0.83 ± 0.09	144 ± 17	100 ± 10	0.68 ± 0.12
Diazepam—28 days→diazepam—1 hr→PTZ	88 ± 9¶	100 ± 7	1.19 ± 0.09¶	127 ± 12	95 ± 12	0.75 ± 0.09

*$p<0.05$ relative to no treatment; †$p<0.01$ relative to no treatment; ‡$p<0.01$ relative to PTZ; §$p<0.05$ relative to diazepam 1 hr before PTZ; ¶$p<0.01$ relative to saline 28 days before diazepam—1 hr→PTZ.
Newman-Keuls, N = 5–10.

vulsant influence of diazepam (1 mg/kg, IP) given one hour before PTZ (40 mg/kg, IP). Due to its brief duration of action (24), the second injection of RO15-1788 was administered only 15 minutes prior to PTZ, i.e., 45 minutes after diazepam (RO—14 days→diaz.—45 min→RO—15 min→PTZ). Controls received either vehicle (two drops of Tween 80 in 10 ml distilled water), a jab with an empty syringe needle or nothing in lieu of the first injection of RO15-1788 or only a single exposure to RO15-1788, its vehicle or needle jab 14 days before being given diazepam and PTZ.

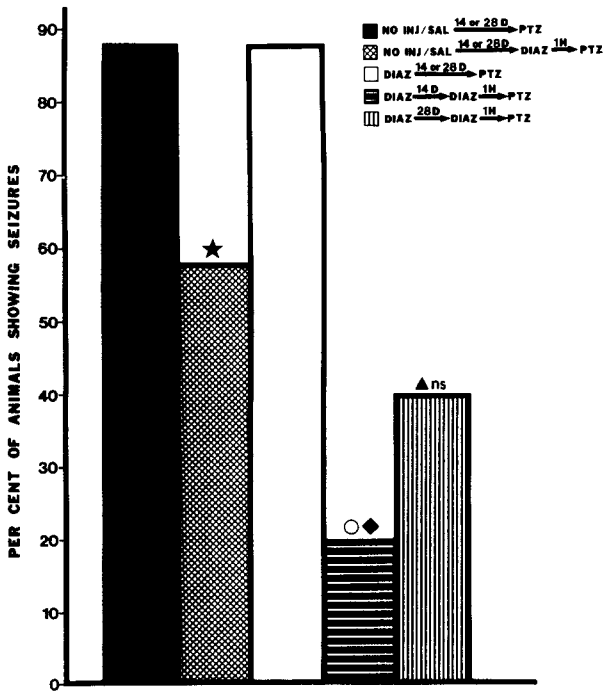

FIG. 1. Effect of spaced diazepam (DIAZ; 0.5 mg/kg, IP) treatments on pentylenetetrazole- (PTZ; 40 mg/kg, IP) induced convulsions. Controls received one of the following treatments: PTZ alone (black bar); a single injection of DIAZ one hour before PTZ (stippled bar); an injection of saline 14 or 28 days prior to the DIAZ treatment an hour before PTZ (stippled bar); DIAZ (white bar) or saline (black bar) 14 or 28 days before PTZ. Experimentals received two injections of DIAZ spaced either 14 (horizontal bar) or 28 (vertical bar) days apart and PTZ one hour after the second DIAZ treatment. ★$p = 0.02$ relative to PTZ; ○$p = 0.04$ relative to diazepam 1 hour before PTZ; ▲$p = 0.009$ relative to PTZ; ◆$p = 0.003$ relative to PTZ. ns—not significantly different from diazepam 1 hour before PTZ; Fishers Exact Test, N = 10–24.

RESULTS

Anticonvulsant Effects of Diazepam and TDS

Figure 1 summarizes the influence of 0.5 mg/kg diazepam pretreatment on the anticonvulsant action of a second injection of this agent 14 or 28 days later. Convulsions were seen in 88% (21/24) of animals receiving PTZ with or without a saline pretreatment. As expected, diazepam administered 1 hour before PTZ (diaz.—1 hr→PTZ) significantly reduced convulsions to 58% (14/24) ($p = 0.02$, Fisher's Exact Test). Administration of diazepam once 14 or 28 days before PTZ (diaz.—14 or 28 days→PTZ) did not alter the incidence of seizures, which remained at 88% (14/16). However, when animals received diazepam twice, at 14 days and again at one hour before PTZ (diaz.—14 days→diaz.—1 hr→PTZ), seizures were reduced to 20% (2/10), i.e., almost ⅓ the level seen when only the second treatment was given ($p = 0.04$ relative to diazepam one hour before PTZ; $p = 0.0003$ compared to PTZ). There was a 40% (4/10) incidence of seizures when the interval between diazepam treatments was extended to 28 days, which was not significantly different from that seen in the 1-hour pretreated animals.

Antistress Effects of Diazepam and TDS

Table 1 summarizes the influence of a 28-day interval between diazepam treatments on PTZ's effect on DOPAC, DA and the ratio of one to the other in the n. accumbens and FC. One-way ANOVA indicated a significant overall difference among the effects of treatments on n. accumbens DA, $F(5,30) = 7.5$, $p = 0.001$. Post hoc comparisons using the Newman-Keuls procedure revealed that PTZ increased DA levels by 42% relative to untreated animals ($p<0.05$) and that this could be prevented by diazepam administered one hour earlier ($p<0.01$). This preventive effect of diazepam on PTZ-induced elevation of DA did not occur in animals receiving a saline injection 28 days before diazepam (saline—28 days→diaz.—1 hr→PTZ) ($p<0.01$ relative to diazepam one hour before PTZ) but was reinstated when another diazepam treatment was substituted for saline (diaz.—28 days→diaz.—1 hr→PTZ) ($p<0.05$). None of the treatments affected n. accumbens DOPAC,

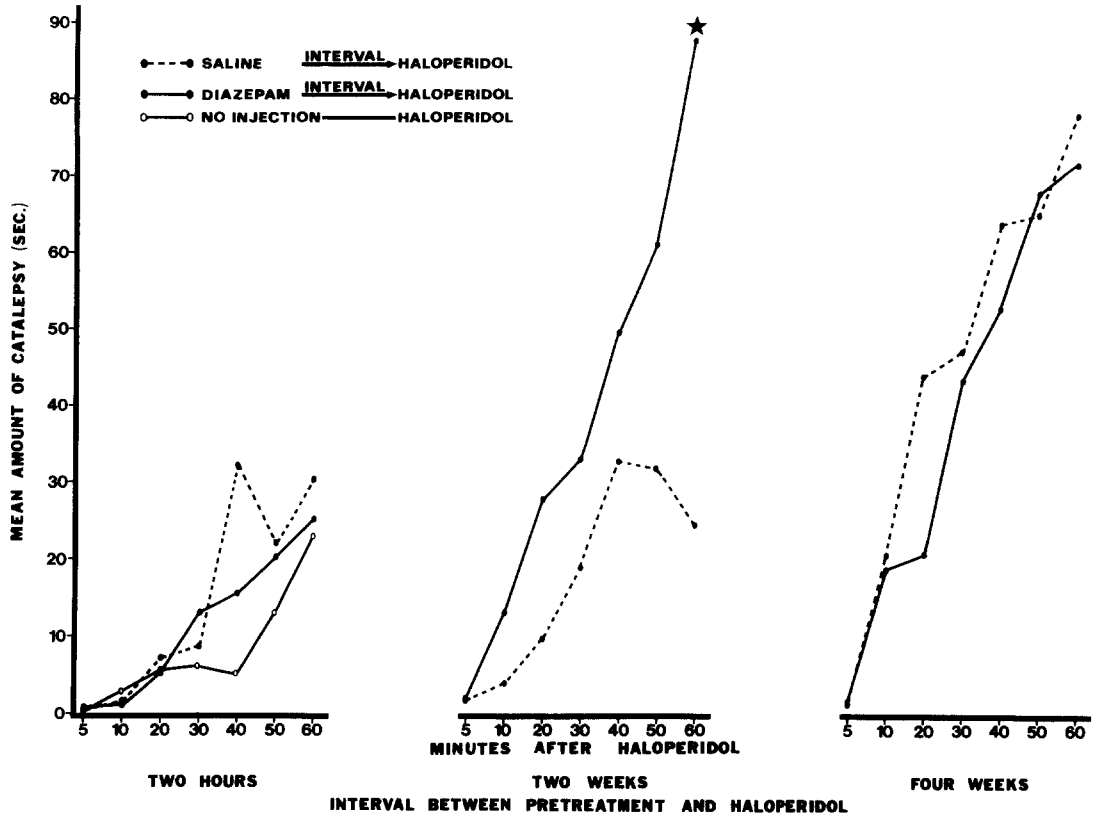

FIG. 2. Effect of diazepam- (2.5 mg/kg) haloperidol (0.4 mg/kg) interval on catalepsy. Diazepam (2.5 mg/kg, IP) or an equal volume of saline was administered either 2 hours, 14 days or 28 days prior to haloperidol (0.4 mg/kg, IP). A series of 2-minute tests of forepaw catalepsy were then conducted between 5 and 60 minutes after haloperidol injection. $\star p = 0.026$, one-way ANOVA. n = 6–10.

possibly because the animals were killed only 10 minutes after PTZ. The coupling of increased DA and no change in DOPAC resulted in significant changes in DOPAC/DA ratios, $F(5,30) = 6.6$, $p = 0.0002$. PTZ decreased this ratio (an often-used measure of DA turnover) relative to untreated animals ($p < 0.01$), an effect which was prevented by diazepam given an hour earlier ($p < 0.01$). In turn, this effect was blocked by saline pretreatment 28 days earlier ($p < 0.01$) and reinstated by diazepam administered at that time as well as one hour before PTZ ($p < 0.05$). A single administration of diazepam 28 days before PTZ had no effect.

The DA pattern in the FC was the same as that observed in n. accumbens. ANOVA was significant, $F(5,29) = 3.44$, $p = 0.01$. Newman-Keuls comparisons indicated increased DA (+123%) after PTZ ($p < 0.01$), which was blocked by diazepam an hour earlier ($p < 0.01$). Again, diazepam was ineffective when preceded by a saline injection 28 days earlier (saline—28 days→diaz.—1 hr→PTZ) ($p < 0.01$). However, in contrast to what was observed in the accumbens, substitution of a second diazepam treatment at that time (diaz.—28 days→diaz.—1 hr→PTZ) failed to significantly reinstate the result seen in animals pretreated only one hour before PTZ. There was no significant difference among groups in terms of frontal cortical DOPAC and due to the variability in this measure, no difference in the DOPAC/DA ratios.

Diazepam and TDS of Neuroleptic-Induced Catalepsy and Ptosis

Figures 2 and 3 summarize the effects of 2.5 mg/kg of diazepam pretreatment on the measurement of catalepsy and ptosis following a subsequent injection of haloperidol. Diazepam administered two hours before haloperidol failed to affect catalepsy or ptosis relative to either no pretreatment or animals that received saline. By contrast, there was a very marked and significant potentiation of both behaviors when the interval between diazepam and haloperidol was extended to two weeks. For example, at 60 minutes after haloperidol, animals pretreated with diazepam 14 days earlier showed a mean catalepsy score of 87 ± 11 seconds while those pretreated with saline had a score of 24 ± 4, which is the same as the groups tested after only a 2-hour interval between treatments [$F(1,17) = 5.85$, $p = 0.026$, for groups; $F(6,102) = 20.38$, $p < 0.00001$, for time of testing and, $F(6,102) = 5.37$, $p = 0.00013$, for group and time interaction]. The total mean ptosis score for animals pretreated with diazepam two weeks earlier was 0.69, compared to 0.1 for those that earlier received saline, $F(1,17) = 6.06$, $p < 0.025$. There were no differences between experimentals and controls when the interval between pretreatment and haloperidol was extended to four weeks. However, this was due to increased catalepsy and ptosis in control-pretreated animals rather than a reduction in these measures in animals receiving diazepam a month earlier.

The results obtained when this experiment was repeated can be seen in Table 2. Haloperidol alone induced much longer periods of catalepsy than were seen in the initial experiment. Nevertheless, diazepam administered a single time 14 days earlier once again significantly increased the duration of haloperidol catalepsy relative to all other groups, including that of diazepam itself given only 2 hours earlier. In contrast to the earlier experiment in which

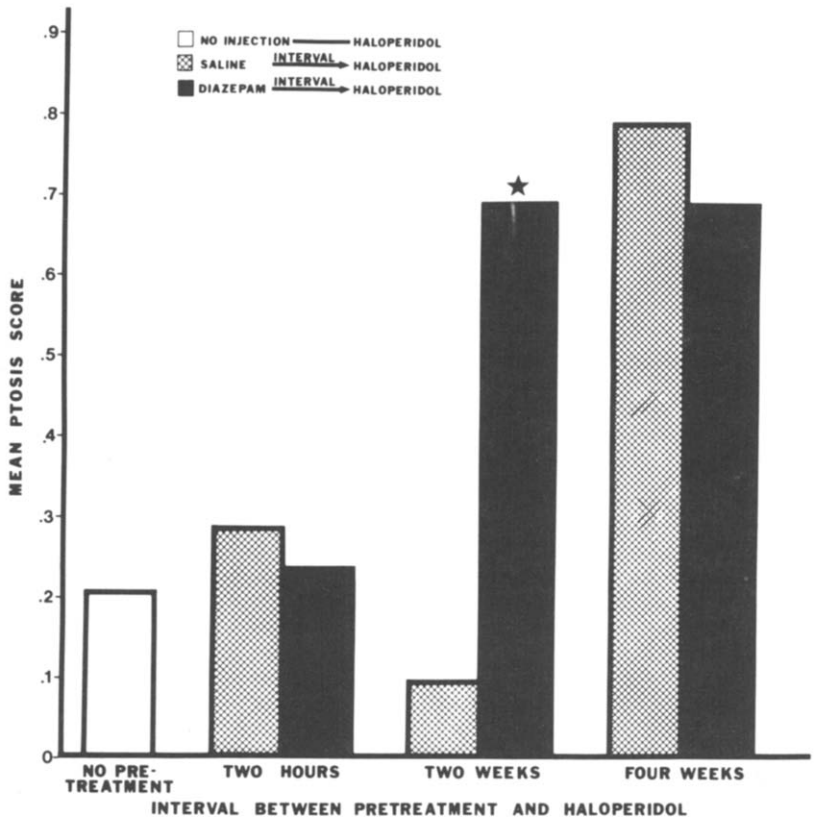

FIG. 3. Effect of diazepam- (2.5 mg/kg) haloperidol (0.4 mg/kg) interval on ptosis. Mean sec. $\star p<0.025$, ANOVA. N = 6–10.

the enhanced catalepsy following diazepam pretreatment 14 days earlier occurred predominantly during the latter part of the 60-minute testing periods (Fig. 2), it was restricted to those tests which took place 10–30 minutes after haloperidol [$F(4,142) = 4.063$, $p<0.005$; all Newman-Keuls comparisons, $p<0.01$ relative to diazepam—14 days→haloperidol].

Administration of RO15-1788 or its vehicle 10 minutes prior to diazepam prevented completely the ability of the latter to induce time-dependent enhancement of haloperidol catalepsy 14 days later [$F(2,78) = 4.954$, $p<0.01$; Newman-Keuls, $p<0.05$]. The effect of RO15-1788 and vehicle did not differ significantly.

As with catalepsy, baseline ptosis scores after haloperidol were also higher than in the initial experiment. Nevertheless, diazepam administered 14 days but not 2 hours earlier, induced a significant, 145% increase in ptosis scores, $F(6,197) = 3.12$, $p<0.01$, during the 10–30 minute tests (Table 3). Pretreatment with RO15-1788 or its vehicle failed to prevent diazepam's action.

The nucleus accumbens DOPAC response to haloperidol was significantly enhanced in animals receiving diazepam 2 hours earlier or diazepam or saline 14 days earlier (Table 4). RO15-1788 or its vehicle prior to diazepam, failed to prevent this effect [$F(7,66) = 17.53$, $p<0.01$; Newman-Keuls, $p<0.05$]. Diazepam pretreatment did not modify the DOPAC response to haloperidol in either the FC or striatum.

TDS and RO15-1788

PTZ alone caused convulsions in 100% of the animals tested (6/6) (Table 5). Diazepam administered an hour earlier reduced the incidence to 12.5% (1/8). The incidence of convulsions remained at this level in the animals which also received RO15-1788 15 minutes before PTZ (1/8—12.5%; $p<0.002$ relative to PTZ, Fisher's Exact Test). In contrast, those receiving two RO15-1788 treatments 14 days apart had a significantly greater incidence of seizures (5/10—50%; $p<0.05$ relative to diazepam—1 hr→PTZ + diazepam—45 min→RO15-1788—15 min→PTZ).

DISCUSSION

The main findings of this study can be summarized as follows:

1) A single treatment with a low (clinically relevant) or moderate dose of diazepam significantly sensitized the anticonvulsant response to a second exposure to the same agent or the cataleptic or ptotic response to a neuroleptic two weeks later.

2) Pretreatment with the benzodiazepine antagonist, RO15-1788, similarly sensitized the antidiazepam action of a second exposure to this agent two weeks later.

3) An injection of saline one month earlier overcame completely the ability of diazepam to antagonize the increase in nucleus accumbens and FC DA levels which occurs after a convulsant dose of PTZ.

The possibility of a pharmacokinetic explanation of the first two findings needs to be considered. However, it appears unlikely for several reasons. First, rat blood and brain levels of diazepam and its principal active metabolite, N-desmethyldiazepam decline to less than 0.01 μg per ml of blood or g of brain tissue by five hours after treatment with a diazepam dose 2–10 times those used in our experiments (20). Second, rat brain concentrations of some benzodiazepines have been shown to vary widely even after administration of agents with very similar ED_{50}'s against PTZ-

TABLE 2

ENHANCEMENT OF HALOPERIDOL- (0.4 mg/kg, IP) INDUCED CATALEPSY BY ADMINISTRATION OF DIAZEPAM (2.5 mg/kg, IP) TWO WEEKS BEFORE AND ITS PREVENTION BY RO15-1788 (2 mg/kg, IP) OR STRESS

Group		Minutes After Haloperidol Injection					
		10	20	30	40	50	60
I.	Haloperidol (10)	60.4 ±13.8	55.7 ±15.0	73.1 ±15.2	82.2 ±11.7	91.0 ±12.5	76.0 ±12.6
II.	Saline—2 hr→Haloperidol (10)	63.4 ±15.9	51.3 ±15.2	52.2 ±12.5	69.3 ±14.0	91.8 ±11.7	88.4 ±12.7
III.	Diazepam—2 hr→Haloperidol (10)	41.6 ±14.2	33.6 ±10.6	93.7 ±13.4	68.9 ±14.4	79.2 ±15.0	90.6 ±11.7
IV.	Saline—2 weeks→Haloperidol (10)	61.0 ±15.9	56.5 ±16.9	69.0 ±15.1	98.6 ±9.1	82.4 ±13.8	90.1 ±12.4
V.	Diazepam—2 weeks→Haloperidol*† (9)	93.0 ±14.2	99.3 ±11.4	99.0 ±11.3	101.6 ±8.5	103.3 ±8.4	94.2 ±11.1
VI.	RO15-1788—10 min→Diazepam 2 weeks→Haloperidol (10)	35.8 ±14.4	59.6 ±15.1	88.6 ±11.8	88.4 ±11.3	82.1 ±13.0	93.6 ±10.7
VII.	Vehicle—10 min→Diazepam 2 weeks→Haloperidol (8)	64.1 ±16.8	66.8 ±16.5	83.6 ±17.8	83.4 ±15.0	82.0 ±14.9	109.6 ±7.0

Values represent the mean ± S.E. of catalepsy measured in seconds. Numbers in parentheses = N.

*One-way ANOVA groups I–V for 10–30-min catalepsy tests, $F(4,142) = 4.063$, $p<0.005$; Newman-Keuls comparisons all $p<0.01$ relative to Group V (Diazepam—2 weeks→Haloperidol).

†One-way ANOVA groups V–VII for 10–30-min catalepsy tests, $F(2,78) = 4.954$, $p<0.01$; Newman-Keuls comparisons both $p<0.05$ relative to Group V (Diazepam—2 weeks→Haloperidol).

induced seizures (13), suggesting that this is not the critical variable in determining the anticonvulsant effects of these compounds. Finally, the fact that no effect on haloperidol-induced catalepsy or ptosis was seen when animals were tested two hours after diazepam (a time when both drug and metabolite levels would be *relatively* high) but was obtained with a two-week diazepam-haloperidol interval (when no residual benzodiazepine would be expected) (Figs. 2 and 3, Tables 2 and 3) provides a compelling argument against any obvious pharmacokinetic explanation of our results. Instead, this finding indicates that diazepam-induced sensitization develops (i.e., grows) with the passage of time following acute treatment as has previously been found with antidepressants (4, 6, 14).

The very brief duration of action of RO15-1788 (24) also argues against explaining its sensitizing effects in terms of pharmacokinetics. Others have likewise reported persisting effects of benzodiazepines which cannot readily be explained pharmacokinetically (44,45).

The induction of sensitization is thought to require the presence of a potentially threatening or stressful stimulus (2) and we have argued that drugs provide such a stimulus by virtue of their being foreign substances to the organism (1,2). The similarity between the sensitizing effect of two spaced treatments with RO15-1788 and only one injection of RO15-1788 preceded two weeks earlier by vehicle or a needle-jab points in this direction by suggesting that the major influence of this agent in initiating sensitization was as a stressor. Some of the pharmacologic actions of RO15-1788 may also resemble those of a stressor since both this compound and its vehicle significantly prevented the ability of diazepam to induce TDS to the cataleptic effects of haloperidol, whereas

TABLE 3

ENHANCEMENT OF HALOPERIDOL- (0.4 mg/kg, IP) INDUCED PTOSIS BY DIAZEPAM (2.5 mg/kg, IP) ADMINISTERED TWO WEEKS EARLIER

	Ptosis Score Mean ± S.E.
Haloperidol (10)	0.83 ± 0.192
Diazepam—2 hrs→Haloperidol (10)	1.50 ± 0.147
Saline—2 hrs→Haloperidol (10)	1.10 ± 0.222
Diazepam—2 weeks→Haloperidol (10)	2.03 ± 0.269*
Saline—2 weeks→Haloperidol (10)	1.43 ± 0.238
RO15-1788—10 min→Diazepam—2 weeks→Haloperidol (10)	1.87 ± 0.298†
Vehicle—10 min→Diazepam—2 weeks→Haloperidol (8)	2.00 ± 0.351*

ANOVA, 10–30 minutes after haloperidol, $F(6,197) = 3.12$, $p<0.01$. Newman-Keuls, *$p<0.05$, †$p = 0.05$, relative to haloperidol.

Numbers in parentheses = N.

TABLE 4

ENHANCEMENT OF THE NUCLEUS ACCUMBENS DOPAC RESPONSE TO HALOPERIDOL (0.4 mg/kg, IP) IN RATS PRETREATED WITH A SINGLE INJECTION OF DIAZEPAM (2.5 mg/kg, IP) OR SALINE TWO WEEKS EARLIER

	μg/g
No Treatment (6)	1.37 ± 0.16
Haloperidol (10)	3.29 ± 0.14*
Diazepam—2 hr→Haloperidol (10)	4.00 ± 0.19†
Saline—2 hr→Haloperidol (10)	3.59 ± 0.17
Diazepam—2 weeks→Haloperidol (10)	4.12 ± 0.23†
Saline—2 weeks→Haloperidol (10)	4.25 ± 0.21†
RO 15-1788—10 min→Diazepam 2 weeks→Haloperidol (10)	4.16 ± 0.25†
Vehicle—10 min→Diazepam 2 weeks→Haloperidol (8)	4.18 ± 0.13†

ANOVA, $F(7,66) = 17.53$, $p<0.001$.

Newman-Keuls, *$p<0.01$ relative to No Treatment; †$p<0.05$ relative to haloperidol.

Number in parentheses = N. Sacrifice occurred 60 minutes after haloperidol.

neither affected TDS of ptosis.

The finding that diazepam-induced TDS of catalepsy and ptosis after haloperidol was manifest at different times of the testing period in our initial and repeat experiments may relate to the substantially greater effect of haloperidol in inducing these behaviors in nonpretreated controls in the second experiment. On the other hand, it is notable that sensitization sometimes occurs as an earlier response to a subsequent drug or stressor (25, 46, 48) (Table 2) and at other times as a more prolonged reaction to such a stimulus (17, 26, 51) (Fig. 2). The reason for this dichotomy is not apparent, although it is widespread and has been observed across species, test stimuli and physiological systems.

We did not anticipate the earlier response to haloperidol when the diazepam-haloperidol experiment was repeated and therefore, sacrifice for out biochemical measurements did not take place until the end of the testing period (60 minutes after haloperidol), by which time sensitization of catelepsy and ptosis were complete. The results were interesting nevertheless. Diazepam-pretreated groups—including those receiving RO15-1788 prior to diazepam—all had significantly greater levels of DOPAC in the nucleus accumbens after haloperidol relative to haloperidol animals not receiving a pretreatment. However, as was the case with the four-week pretreatment-haloperidol intervals in Figs. 2 and 3, saline pretreatment—in this instance 14 days earlier—also significantly enhanced the action of haloperidol. These data confirm previous behavioral findings indicating that a single saline pretreatment can potentiate the actions of neuroleptics administered weeks later (7). Collectively, they suggest that stressors and diazepam can each exert a similar long-term, sensitizing influence on a spectrum of neuroleptic activities. This effect of diazepam may be a consequence of its foreign/stressful properties rather than any pharmacological action since it is not selectively prevented by pretreatment with the benzodiazepine receptor antagonist, RO15-1788. In any event, our findings could be interpreted as suggesting that individuals with a history of stress or earlier exposure to benzodiazepines may be more reactive to many of the actions of neuroleptics. This suggestion is consistent with reports that benzodiazepines may allow reduction of neuroleptic dosages in the treatment of schizophrenia, mania and other psychoses (8).

TABLE 5

THE EFFECT OF TWO INJECTIONS OF RO15-1788 (RO; 1.5 mg/kg) SPACED TWO WEEKS APART ON THE ABILITY OF DIAZEPAM (DZ; 1 mg/kg) TO BLOCK CONVULSIONS AFTER PENTYLENETETRAZOLE (PTZ; 40 mg/kg)

	No. Convulsions/ No. Rats
PTZ	6/6
DZ—1 hr→PTZ	1/8*
DZ—45 min→RO—15 min→PTZ	1/8*
RO—2 weeks→DZ—45 min→RO—15 min→PTZ	5/10†
VEH—2 weeks→DZ—45 min→RO—15 min→PTZ	3/7
INJ—2 weeks→DZ—45 min→RO—15 min→PTZ	3/7
RO—2 weeks→DZ—1 hr→PTZ	2/7
VEH—2 weeks→DZ—1 hr→PTZ	2/7
INJ—2 weeks→DZ—1 hr→PTZ	2/7

VEH = Vehicle; INJ = Injection with an empty syringe needle.

*$p = 0.002$ relative to PTZ; †$p = 0.05$ relative to DZ—1 hr→PTZ + DZ—45 min→RO—15 min→PTZ, Fisher's Exact Test.

The fact that a model for assessing anxiolytic [antagonism of PTZ-induced convulsions (32)] effects showed evidence of TDS after acute diazepam administration suggests the possibility that this action may also progress with the passage of time during clinical treatment with benzodiazepines. If so, our results might imply that at least some clinical effects cannot be attributed to the acute physiological actions of these agents per se but rather to either a progressive intensification of such acute actions or to an alteration which evolves as a function of time after acute treatment. Interestingly, work with both animal models and clinical trials has shown that the anxiolytic effects of benzodiazepines are more marked after about a week of daily treatment (19, 33, 39). It has traditionally been assumed that this results from a progressive decrease in the sedative effects of benzodiazepines (33). In light of our results, such findings must now also raise the question of whether daily benzodiazepine treatment is really necessary to achieve anxiolytic effects or whether subsequent administrations merely serve to "recall" or reveal a time-dependent (repeated-treatment independent) sensitization of the anxiolytic process requiring only an acute treatment in order to be set in motion. This question could easily be addressed clinically by comparing the anxiolytic effect achieved by two benzodiazepine treatments spaced one week apart with that observed after daily treatment over the same time period. The possibility must also be entertained that other, often unwanted effects of benzodiazepines, such as sedation may also sensitize with time after acute treatment. It is relevant to note that in a very recent study with patients suffering from major depression, we found that therapeutic effects grew significantly with time after acute chlorimipramine treatment (36).

A number of interesting findings emerged when we viewed the effects of PTZ and diazepam on DA levels and utilization in cortical and limbic areas. In contrast to the enhancement in DA neuronal activity in nucleus accumbens and/or frontal cortex reported after other stressors (18, 29, 49). PTZ significantly increased DA levels in both regions while decreasing the DOPAC/DA ratio (singificant only in the nucleus accumbens), strongly suggesting a decrease in DA activity. Pretreatment with 0.5 mg/kg diazepam one hour earlier significantly prevented these effects, i.e., DA levels were reduced and the DOPAC/DA ratio was increased relative to PTZ. This effect of diazepam on stressor-induced changes in DA activity is opposite to that seen when stressors increase DA function (18,29). However, taken together with these data, our finding suggests that benzodiazepines may

exert a modulatory or homeostatic (i.e., normalizing) influence on subsequent stressors regardless of whether these increase or decrease DA activity. It is possible that much of the change in frontal cortex DA levels observed after PTZ could have occurred in NE-containing neurons (22). However, the likelihood of this possibility is diminished since similar changes were also observed in the nucleus accumbens. Although the reason why PTZ decreased DA neuronal activity is unknown, one possibility is that the type of paroxysmal neuronal excitation typically associated with seizures led to a postictal cessation of firing such as is seen during depolarization blockade (12) or the administration of agents such as gammabutyrolactone (52). In the latter case, there is a buildup in DA levels as was observed here (52). If this hypothesis is correct, then the inhibitory, GABA-like action of diazepam might have normalized DA activity by preventing the cessation of neuronal firing. Consistent with what might be expected during cessation of DA neuronal discharge, for several minutes following convulsions, PTZ-treated rats remain immobile and do not appear to attend to sudden, loud noises in the environment.

It is particularly intriguing that when diazepam administered one hour before PTZ was itself preceded by a single saline injection 28 days earlier (saline—28 days→diaz.—1 hr→ PTZ), it lost the ability to reverse the effect of PTZ on DA levels (but not its anticonvulsant activity) in the accumbens and FC and the DOPAC/DA ratio in the accumbens. Since saline was administered as an isotonic solution in a reasonable volume (1 ml/kg), it seems likely that its ability to prevent the anti-PTZ effects of diazepam on DA activity was due to the stress associated with its injection rather than saline per se.

Proceeding on this assumption, we repeated our basic experiment in the FC, this time exposing rats to either the "mild" stress of a single jab with an empty syringe needle 1 hour–28 days before diazepam, or a more severe stressor—2 hours of immobilization by wrapping—28 days prior to diazepam. We also went further and measured the effects of our treatments on plasma corticosterone in the same animals. The milder stressor failed to antagonize diazepam's action on PTZ-induced changes in frontal cortical DA. However, preexposure to the stress associated with immobilization completely replicated our earlier findings with the saline injection. PTZ alone elevated DA levels to 232% of the control value. This was significantly reduced to 140% of control by diazepam administered one hour before PTZ. In turn, exposure to immobilization one month earlier overcame entirely the anti-PTZ influence of diazepam and brought DA levels back to where they had been after PTZ alone, i.e., 243% of the control value (3). In contrast to what was observed in the FC, *both* the needle-jab stressor 1–28 days earlier and immobilization completely reversed the influence of diazepam on PTZ-induced elevation of plasma corticosterone. Moreover, up to a point, the antidiazepam effect of the stress associated with a needle-jab grew with the passage of time, i.e., it showed TDS. These findings provide still further substantiation of the idea that a single stressor, remote in time, can exert a powerful influence on benzodiazepines as it does on some neuroleptics (7) (Figs. 2 and 3 and Tables 2 and 3), stimulants (5,41) local anesthetics (submitted) and DA agonists (2). Although this is the first report of which we are aware demonstrating that stress can have a very long-lasting influence on benzodiazepine actions, there are several indications of an antibenzodiazepine effect of stress in the short term (9,50). For example, Treit (50) found "tolerance" to the inhibitory effect of diazepam on conditioned defensive burying when the shock level was raised on the prod used to induce this behavior.

We have repeatedly demonstrated long-term TDS in behavioral situations after acute stressors (3,5) and this may have occurred in the present instance, possibly reducing diazepam binding to benzodiazepine receptors as has been shown after some stressors (11,31) and thereby preventing its anti-PTZ effect. However, we must caution that at this point such a possible mechanism is entirely speculative, since only the immediate effects of stressors on benzodiazepine receptors have been examined to date. Whatever the mechanism, this finding may also be important in its own right, since it appears to provide the first neurochemical evidence that an acute stressor can induce very long-lasting effects on mesolimbic and mesocortical DA activity. Stress-induced sensitization may also have accounted for the finding that saline-pretreated animals showed the same enhanced responses to haloperidol (Fig. 2) as those pretreated with diazepam when there was a four-week interval between treatments, since we have earlier shown long-term TDS to neuroleptic-induced catalepsy after saline injection (7). Since substitution of diazepam for saline injection reinstated the anti-PTZ action (in the accumbens but not the cortex) of a second diazepam administration one month later (diaz.—28 days→diaz.—1 hr→PTZ) this might suggest that the antistress effects of benzodiazepines can persist for some time. Alternatively, the initial diazepam treatment may merely have served to prevent the stressful effects of injection.

In summary, this study has provided the first evidence that the effects of some benzodiazepines can grow with the passage of time following acute administration. Future work in this area will need to determine whether these findings extend to similar compounds and whether they can be mimicked by GABA agonists. At present, they lend further credence to our hypothesis that TDS after acute drug treatment reflects a general principle of nervous functioning (1, 2, 6) which might be applicable to all drugs (and dosages) perceived by the organism as foreign substances. The implications of this hypothesis for both a revision of the most basic principles of pharmacotherapy as well as increasing our knowledge of how drugs act on the nervous system may be considerable (1,2).

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