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Therapeutic and Hypothermic Properties of Diazepam Altered by a Diazepam-Chlorpromazine Association

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TAUKULIS, H K AND L D BRAKE *Therapeutic and hypothermic properties of diazepam altered by a diazepam-chlorpromazine association* PHARMACOL BIOCHEM BEHAV **34**(1) 1–6, 1989 —Rats were injected (IP) with diazepam (2 5 mg/kg) and chlorpromazine (10 0 mg/kg) with a 30-min interval between the two injections After 10–12 repeated drug pairings of this type, the thermic, muscle relaxant, and anxiolytic responses of the animals to diazepam alone were tested These tests revealed 1) an enhanced hypothermia (rectal temperature), 2) an attenuated muscle relaxant effect (inclined plane test), and 3) a potentiated anxiolytic effect (plus-maze test) Although various interdrug associations have previously been demonstrated using other measures of conditioning, this is the first instance in which changes in the therapeutic effects of a drug (in this case, muscle relaxation and anxiety reduction) have been obtained with this procedure

Interdrug associations Diazepam Chlorpromazine Inclined plane Plus-maze Hypothermia Anxiolytic response Muscle relaxation

WHEN two psychoactive drugs are administered sequentially to rats on repeated occasions, changes in the physiological and/or behavioral effects of the first drug may be conditioned Conditional responses obtained thus far have included shifts in drug-induced thermic effects (35–37), heart rate (26,41), stomach emptying (13), and learned flavor aversions (5, 12, 17, 25, 27–29).

Despite the variety of drug effects that have been studied, none of the experiments so far have dealt with the therapeutic effects of a drug, i e., those applications for which the drug is most frequently prescribed The experiments described here provide the first demonstration that such effects are malleable through a process of interdrug conditioning

Diazepam (DZ), a 1,4-benzodiazepine, is employed mainly for its four major actions muscle relaxant, anxiolytic, anticonvulsant, and sedative It is sometimes combined with neuroleptics like chlorpromazine or haloperidol to counteract the akathisia or acute dystonic reactions that these agents may induce (2) Clinical reports (14) and laboratory studies (33) have suggested that a diazepam-neuroleptic combination may prove beneficial for the alleviation of psychotic symptoms in certain types of patients No adverse effects of such a compound have so far been reported in human subjects, and the only interaction of concern appears to be an additive sedation (2). At the same time, animal studies of associative drug effects have shown that cues signalling a neuroleptic can elicit conditional responses (6, 22, 23, 36) For all of these reasons, diazepam and chlorpromazine were chosen to serve as the paired drugs in the present study, with the focus on potential changes in the rats' responses to the benzodiazepine The experiments to be described concentrated on two of the therapeutic actions of diazepam, muscle relaxation and anxiety reduction, as well as on one of its minor effects, hypothermia.

Taukulis (36) demonstrated a conditional hyperthermia in rats in response to atropine sulfate when this drug had previously been paired with chlorpromazine hydrochloride (CPZ) It was argued that this upward thermic shift was an indication of a learned association between the two drug states Animals that had received the drugs in reverse order (i e , chlorpromazine preceded atropine by 30 minutes) did not exhibit this effect, thus ruling out the possibility that a nonassociative interaction of some sort was the cause In Experiment 1 this same measure, rectal temperature, was employed as an index of association between diazepam and chlorpromazine The purpose of this study was simply to provide preliminary evidence, using a proven method of detection, that this drug combination is capable of yielding a conditional response

Experiment 2a sought a conditional change in diazepam's muscle relaxant property. Muscle tone was measured via the inclined plane technique [e.g., (18)]. A rat is placed upon a board that is gradually tilted until the animal begins to slide. Diazepam will normally reduce the slope that the animal is able to tolerate before slipping.

Experiment 2b was designed to test the effects of diazepam-chlorpromazine pairings in one particular animal model of anxiety, the plus-maze test. While many animal models have been devised that purport to serve as analogues of human anxiety, few can selectively screen for anxiolytic properties alone [see Treit (40) for a review of these methods and the problems associated with them]. However, a recently devised technique utilizing an elevated plus-maze has shown considerable promise as a sensitive measure of a drug's anxiolytic potential (1, 19, 20). The cross-shaped maze has two enclosed arms and two open arms. A rat is placed at the center of the maze and is observed for five minutes. An anxiolytic drug like diazepam will increase the amount of time that the rats will spend in the open arms as well as the percentage of open arm entries. Drugs that have sedative effects, but are not specifically anxiolytic, will simply reduce the overall activity level of the animals without altering the frequency or duration of excursions into the open arms.

GENERAL METHOD

Subjects

Sixty male Long-Evans rats were obtained from Charles River Canada at a weight range of 50–75 g and were raised in the laboratory until they had attained an average weight of 338 g (Experiment 1) or 487 g (Experiment 2). They were maintained in translucent plastic cages (Hazleton HP 301) with wire tops. Food was provided ad lib, and water was available at all times except as indicated below. The animal holding room was maintained at a constant 22–24°C with a 14/10-hr light/dark cycle, it was here that all experimental manipulations were performed.

EXPERIMENT 1: RECTAL TEMPERATURE TEST

Materials

Rectal temperatures were obtained with a thermistor probe (YSI No. 423) linked to a Cole-Parmer digital thermistor thermometer capable of a 0.1°C resolution (Model 8110-20 or Model C-8522-10). The drugs used were diazepam (Valium injectable, Roche) and chlorpromazine hydrochloride (Sigma Chemical Company).

Procedure

Thirty-six rats were employed in this study. For this experiment alone the animals were deprived of water for an 8-hr period each day (from 0900 to 1700 hr). The removal of water bottles served to facilitate the extraction of the rats from their cages during experimental procedures. It also helped to decrease the variability in rectal temperature that occurs as a by-product of fluid consumption (Taukulis, unpublished observation).

The experiment was comprised of three phases: familiarization, treatment, and testing. Familiarization involved a six-day period during which each animal was given a daily intraperitoneal (IP) injection of physiological saline (0.5 ml per injection) and exposure to the temperature measurement procedure. In the Treatment Phase, animals were given two double-injection sessions per week over a period of ten weeks. During one of these weekly sessions, both injections contained physiological saline. During the second weekly session, each animal received a pair of injections, spaced 30 min apart, appropriate to its experimental group (n = 12 in each case). Group DZ-CPZ was administered diazepam (2.5 mg/kg) followed 30 min later by chlorpromazine (10 mg/kg). Group CPZ-DZ received the same drugs in reverse order, and Group DZ was given either DZ-saline (n = 6) or saline-DZ pairings (n = 6) (saline was administered in a volume of 2 ml/kg per injection).

During weeks 1, 4, 7, and 10, rectal temperatures were taken immediately before the first injection and 60 min after the second injection on both treatment days. On each of these days, the following procedure applied. All animals were weighed at least 30 min prior to any temperature reading. At the start of a session, each animal was removed from its home cage and held lightly at the base of the tail by the experimenter who was wearing a padded leather glove. The thermistor probe was inserted into the rectum to a depth of 6 cm and held there until the thermometric reading stabilized (defined as a 10-sec period of no change). Immediately after removal of the probe, an IP injection of drug or saline was administered, followed 30 min later by a second injection. Sixty minutes after this second injection, temperature readings were again taken. The animals spent the time between injections and temperature measurements in their home cages.

The Test Phase of the experiment began 7 days after the last treatment session. All animals were given only one injection per day, and this injection was the same across all four groups. On Test Day 1 saline (0.5 ml/kg) was injected, and on Test Day 2 the injection contained diazepam (2.5 mg/kg). Temperature readings were obtained immediately prior to and at intervals of 30, 60, 120, 180, and 240 min following each of these injections.

EXPERIMENT 2A: INCLINED PLANE TEST FOR MUSCLE RELAXATION

Apparatus

The device used to measure muscle tone consisted of a cork board measuring 45.5 × 60.5 cm and a large protractor. The board was raised manually with its shorter edge resting against a wooden brace affixed to the surface of a table. Its longer edge moved along the surface of the protractor against which the angle of incline could be determined.

Procedure

Twenty-four rats were employed in this study. As in Experiment 1, the procedure involved familiarization, treatment, and testing phases. The Familiarization Phase consisted of an 11-day period during which the animals were weighed and handled. During each of the last five days of this phase, every animal was given one 0.5 ml saline injection. The Treatment Phase consisted of a 10-week period during which the animals experienced two injection sessions per week. During one of these weekly sessions, all animals were given two saline injections (one 0.5 ml/kg and one 2.0 ml/kg injection, equivalent to the volumes in which the drugs were administered). During the other weekly session, Group DZ-CPZ (n = 8) received diazepam (2.5 mg/kg) followed 30 min later by chlorpromazine (10 mg/kg). Group CPZ-DZ (n = 8) received the same doses of these drugs in reverse order, and Group DZ (n = 8) received either diazepam-saline or saline-diazepam pairings (saline = 2.0 ml/kg). All injections were IP.

The Test Phase began 6 days after the tenth drug treatment session. Animals in all groups were injected with diazepam (2.5 mg/kg) or saline (0.5 ml/kg) and were subjected to the inclined plane test 30 min thereafter. Half the animals in each group were tested with diazepam first and then, 3 days later, were tested with saline. The remainder were tested with saline first followed by a test with diazepam. Each animal was placed on the center of the plane with its head facing upwards. The plane was then raised at

a rate of 2 degrees/second until the animal was unable to maintain its position and slipped off the board. If an animal walked forward to the top of the plane or to either side, the plane was lowered and the trial was begun again This procedure was repeated five times, and the average degree of incline at which the animal slid to the table was calculated

EXPERIMENT 2B PLUS-MAZE TEST FOR ANXIOLYTIC EFFECT

Apparatus

A plus-maze was constructed which conformed closely to that described by Pellow and File (19) It had four arms (50 cm long by 11 cm wide) The two "open" arms opposed each other and were merely boards with no sides, whereas the opposing "enclosed" arms were surrounded by unpainted wooden walls 40 cm high The 11-cm square center of the maze served as the "start" area for each animal on test trials. The entire maze was elevated to a height of 84 cm (This was the only major deviation from the Pellow and File prototype, their maze was elevated to a height of only 50 cm) A 76×61 cm "open field" (a wooden table surrounded by 30 cm high cardboard walls) was used as a preliminary holding area where each subject was placed prior to its trial in the plus-maze

Procedure

During the two weeks following the Test Phase of Experiment 2a, these same animals were given additional conditioning trials of the same type that they had received in the previous Treatment Phase Each week the rats experienced one saline-saline session and one drug treatment session corresponding to their group designations

Testing began three days after the last drug treatment session Each rat was removed from its home cage, injected with either diazepam (2 5 mg/kg) or saline (0 5 ml/kg), and then replaced. Twenty-five minutes later, it was placed into the open field for a 5-min "arousal" period. [Pellow and File (20) found that the addition of this procedure increased the overall activity of the rats once they entered the plus-maze.] Immediately thereafter, it was placed on the center square of the plus-maze, its body oriented towards one of the open arms. A single observer seated approximately 1 0 m from the end of one of the open arms counted the number of arm entries and measured the duration of stay in each arm An animal was considered to have entered an arm when all four paws were within the arm. Partial exits (e.g , two paws outside the arm) followed by re-entry into an arm were *not* counted as arm entries. However, a timer was activated whenever the animal had all four paws within an arm without regard for its pattern of exit and entry Half the animals in each group were tested with diazepam and then, three days later, with saline The remainder were tested with saline first followed by diazepam after a three-day interval

RESULTS

Experiment 1

The thermic responses of all three groups to a single test administration of diazepam are shown in Fig. 1. The points on the graph represent mean changes from baseline temperatures taken immediately prior to the DZ injection Although all groups exhibited temperature losses over the four-hour period, Group DZ-CPZ developed a substantially greater hypothermia within 60 min after DZ administration The statistical significance of this difference was determined by an ANOVA using the maximum shift in temperature from the predrug baseline as a data point for each rat This yielded, $F(2,33) = 5\ 53$, $p<0.01$ A similar analysis

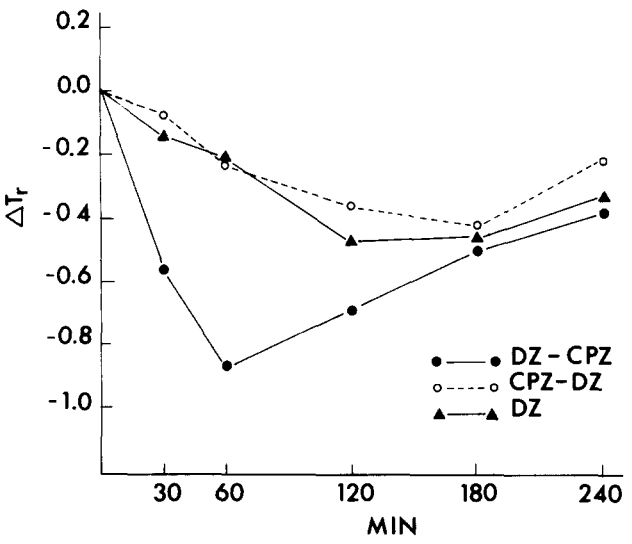

FIG 1 Results of Experiment 1 All animals were injected with 2 5 mg/kg of diazepam Mean changes in rectal temperature (°C) from a predrug baseline are depicted for each group of subjects

of temperature shifts on the previous test day during which saline and not DZ was injected showed that no significant differences among the groups had been detected, indicating that other cues such as those attending the injection procedure were not responsible for the conditional hypothermia observed in Group DZ-CPZ on the following day

Experiment 2a

The average angle at which animals from each group lost the ability to maintain their position at the center of the inclined plane is shown in Table 1. Averages for both diazepam and saline trials are illustrated An analysis of variance performed on the *difference* in angle between the diazepam and saline conditions for each subject yielded, $F(2,21) = 6.36$, $p<0\ 01$ Subsequent comparisons indicated that Group DZ-CPZ differed from Groups CPZ-DZ ($p<0\ 05$) and DZ ($p<0\ 01$), but the latter two groups did not differ from one another ($p<0\ 20$)

All groups showed diminished muscle tone after they had been injected with diazepam. However, this effect was weakest in Group DZ-CPZ Apparently the diazepam-chlorpromazine pairings had served to attenuate the muscle relaxation normally induced by the benzodiazepine. Note that all groups had received equal experience with diazepam during the treatment phase, so tolerance to the drug's effects cannot be implicated as a cause of this attenuation.

Experiment 2b

The results of Experiment 2b are presented in Tables 2 to 4

TABLE 1

EXPERIMENT 2a

Group	Diazepam	Saline
DZ-CPZ	55 0 (1 7)	63 6 (1 1)
CPZ-DZ	50 0 (1 3)	63 0 (1 6)
DZ	44 6 (1 5)	60 4 (1 6)

Mean angle of inclined plane (±S E M) tolerated by each group 30 min after a diazepam or saline injection

TABLE 2

EXPERIMENT 2b

Group	Diazepam		Saline	
	Open Arms	Enclosed Arms	Open Arms	Enclosed Arms
DZ-CPZ	152 1 (21 5)	68 5 (17 5)	4 5 (3 0)	223 6 (13 3)
CPZ-DZ	74 0 (19 3)	168 4 (17 4)	13 6 (8 3)	220 0 (13 8)
DZ	88 8 (20 4)	154 1 (25 3)	9 5 (6 3)	207 3 (11 0)

Mean number of seconds (S E M in parentheses) spent by each group in the open and enclosed arms of a plus-maze during a 5-min interval beginning 30 min after a diazepam or saline injection

Looking first at the mean amounts of time each group spent in the two types of arms after a diazepam injection compared with a saline injection (Table 2), it can be seen that all three groups exhibited a shift towards longer periods in the open arms However, this shift was greatest for Group DZ-CPZ, indeed, only these animals spent more time in the open arms than in the enclosed arms For statistical purposes, the mean percentage of time spent in the open arms relative to the time spent in both types was calculated for all groups An analysis of variance performed on these percentages yielded, $F(2,21) = 6\ 47$, $p < 0\ 01$ Subsequent comparisons revealed that Group DZ-CPZ spent a greater percentage of time in the open arms relative to each of Groups CPZ-DZ and DZ ($p < 0\ 01$) while the latter two did not differ from one another A similar analysis of the data obtained when saline rather than diazepam was injected yielded no group differences

The mean number of entries into each type of arm obtained for each group is shown in Table 3 Here, too, a comparison of the percentage of entries into the open arms relative to the total number of entries following a diazepam injection yielded statistical significance, $F(2,21) = 6\ 76$, $p < 0\ 01$ All groups entered the open arms more frequently after a diazepam injection than they had after a saline injection, but only Group DZ-CPZ exhibited a higher percentage of open arm entries relative to its total arm entries This difference emerged not because Group DZ-CPZ entered the open arms more frequently than the other groups, but because they entered the enclosed arms *less* frequently As a result,their total number of entries tended to be lower than those of the other groups, as shown in Table 4 However, an ANOVA performed on these totals indicated that this difference was not statistically reliable, $F(2,21) = 1\ 56$, $p > 0\ 20$ Additional ANOVAs comparing the total number of entries after saline and the total time spent in both types of arms under each injection condition also yielded no group differences

GENERAL DISCUSSION

The results of Experiments 2a and 2b constitute the first demonstration that an interdrug conditioning procedure can alter a drug's therapeutic properties The pairing of diazepam with chlorpromazine effected an attenuation of diazepam-induced muscle relaxation as measured by the inclined plane test and yielded an enhancement of the drug's action in a putative model of anxiety, the elevated plus-maze Thus, the same interdrug association can simultaneously increase and diminish different aspects of a drug response

TABLE 3

EXPERIMENT 2b

Group	Diazepam		Saline	
	Open Arms	Enclosed Arms	Open Arms	Enclosed Arms
DZ-CPZ	3 4 (0 7)	2 8 (1 1)	0 3 (0 2)	5 9 (0 7)
CPZ-DZ	3 3 (0 5)	7 0 (1 5)	0 9 (0 5)	6 5 (1 7)
DZ	4 5 (1 2)	5 5 (1 1)	0 5 (0 3)	7 1 (0 6)

Mean number of entries (S E M in parentheses) into the open and enclosed arms of a plus-maze during a 5-min interval beginning 30 min after a diazepam or saline injection

TABLE 4

EXPERIMENT 2b

Group	Diazepam		Saline	
	Time	Number	Time	Number
DZ-CPZ	220 6 (19 0)	6 1 (1 7)	228 1 (11 2)	6 1 (0 7)
CPZ-DZ	242 4 (12 1)	10 3 (1 7)	233 6 (9 4)	7 4 (0 9)
DZ	242 9 (10 1)	10 0 (2 2)	216 8 (8 4)	7 6 (0 5)

Total number of seconds spent in both types of arms and total number of crossings into both types of arms (means with S E M in parentheses) during a 5-min interval beginning 30 min after a diazepam or saline injection

In the experiments described here, relatively high doses of diazepam (2 5 mg/kg) and chlorpromazine (10 mg/kg) were employed However, the enhancement of diazepam's anxiolytic effect has also been obtained in replications performed in this laboratory with smaller quantities of both drugs Taukulis and Brake (38) paired a lower dose of the benzodiazepine (2 0 mg/kg) with doses of chlorpromazine ranging from 2 5 to 10 0 mg/kg, all combinations yielded enhanced anxiolysis

In comparison with reports from other investigators (19,20) the animals in this experiment tended to be relatively less active in the plus-maze than we might have preferred As Pellow *et al* (19) pointed out, the strain of rat that is used can make a substantial difference in the baseline performance of subjects exposed to this maze In addition, our own experience has been that younger animals will tend to be more active, though the relative differences between groups will remain the same In an experiment very similar to this one, Brake (unpublished manuscript) found that groups of somewhat younger hooded Long-Evans males exhibited means ranging from 12 5 to 18 2 entries into both types of arms following a 2 5 mg/kg diazepam injection In her experiment, the group with DZ-CPZ treatment experience had the *highest* number of total entries relative to CPZ-DZ and DZ control groups but nonetheless exhibited a markedly greater tendency to enter and remain in the open arms Thus, activity level as measured by total arm entries does not appear to be a critical variable in these experiments

The results of these studies are important because they draw attention to a type of drug interaction that has only recently been explored Changes in drug activity resulting from interdrug associations were unknown until discovered by Revusky *et al* (28) These investigators paired pentobarbital and lithium chloride and found that pentobarbital's capacity to bring about a reduction in the palatability of a saccharin solution was thereby markedly reduced Taukulis (35) later found that this pentobarbital-lithium combination will also yield an attenuation of the hypothermia that the barbiturate normally induces when administered to rats Various other drug combinations have subsequently been studied, but in no instance have the experiments been concerned with the clinically beneficial effects of a drug The present experiments provide evidence that these, too, are susceptible to modification by interdrug conditioning

Lett (12) and Taukulis (35) postulated that some of the

alterations of drug action may come about because the drugs elicit a compensatory response in the organism This concept, borrowed from Siegel (31,32), suggests that the first drug of a sequentially administered pair acts as a signal for the effects of the second drug, or more accurately, as a signal for the effects of the drug-drug interaction As a result of repeated experience with the combination, the organism comes to anticipate the consequences of the interaction as soon as it detects the signal drug's effects In the terminology of Pavlovian conditioning, a normal effect produced by a drug pair (for example, peripheral vasodilation leading to core hypothermia) can be considered an unconditional response, or UCR, and the anticipatory reaction (for example, physiological events that will attenuate the hypothermia) can be considered the conditional response, or CR This type of analysis has a good deal of intuitive appeal It has been used to explain numerous instances of conditional responses accruing to environmental cues (e g , a particular cage or a particular time of day) that have been repeatedly and consistently paired with a drug

Although this "conditional compensatory response" model offers a plausible explanation for some conditional phenomena, its usefulness for the analysis of many interdrug conditioning effects is somewhat limited Signal drugs, like diazepam in the present experiments, are unlike environmental signals in that they have unconditional effects of their own which interact with those induced by signalled drugs like chlorpromazine Because the resultant interaction is so complex and often poorly understood, it is impossible to predict what form an appropriate compensatory response might take For example, we could not predict that, in Experiment 1, the diazepam-induced hypothermia would be enhanced rather than attenuated Furthermore, conditional responses in these experiments are indexed as shifts in the normal effects of the signal drug Thus, the observed effects are a combination of the unconditional effects of the signal drug plus the conditional responses that appear as a function of the drug pairings. These difficulties are further compounded by the fact that many conditional responses are similar to rather than the opposite of an organism's unconditional responses to a drug (7,34) and are therefore not compensatory

In light of this complexity, perhaps a sensible direction for future research might involve an analysis of those changes in the pharmacokinetic and/or pharmacodynamic properties of a signal drug that are a product of the interdrug conditioning procedure In the present example, it may be that the DZ-CPZ conditioning process yields a conditional acceleration of the biotransformation of diazepam into its metabolites, an effect elicited by the stimulus properties of the drug itself This could yield a shift in time-effect curves [see Garattini *et al* (8)] so that the peak of the curve for muscle relaxation, for example, might occur earlier in a diazepam-chlorpromazine pairing group than it does in control groups

Alternative (or complementary) explanations for the conditional effects may involve changes in the interaction of diazepam with one or more neurotransmitter systems The task of searching for these neurological underpinnings will be complicated by two factors alluded to earlier 1) diazepam and chlorpromazine are complex drugs in that they affect several neurotransmitter systems (4,9), and 2) their actions upon these neurotransmitter systems are not well understood It is known that DZ binds to certain benzodiazepine receptor sites and thereby facilitates GABAergic transmission in some way that has not yet been definitively established (3, 10, 16) However, it is not clear that its anxiolytic action is related to its effect on GABAergic neurons Its interactions with serotonergic (30) and dopaminergic systems (11, 24, 39) have also been implicated, as well as interactions with adenosine systems (21)

The complexities surrounding such an analysis can be reduced somewhat by replacing chlorpromazine with simpler, more selective agents that share just one of this neuroleptic's many effects For example, diazepam might be paired with the dopamine antagonist pimozide If one of the rat's responses to diazepam changes in the direction noted in the present experiment, then further research involving the dopaminergic system will be warranted It is probable that the three conditional effects observed in these experiments are mediated by different systems, and the use of system-specific drugs in this paradigm may help to elucidate these differences A fourth property of diazepam, its anticonvulsant effect, is likely to be closely linked to the mechanisms responsible for the drug's anxiolytic action This is suggested by evidence indicating a high correlation between the anxiolytic efficacy of various benzodiazepines and their ability to antagonize pentylenetetrazol-induced convulsions (15) Therefore, diazepam-drug associations that yield increases in the former should also potentiate the latter

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