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Pentobarbital, Diazepam, and Ethanol Abolish the Interphase Diminution of Pain in the Formalin Test: Evidence for Pain Modulation by $GABA_A$ Receptors

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FRANKLIN, K. B. J. AND F. V. ABBOTT. *Pentobarbital, diazepam, and ethanol abolish the interphase diminution of pain in the formalin test: Evidence for pain modulation by $GABA_A$ receptors.* PHARMACOL BIOCHEM BEHAV **46**(3) 661-666, 1993. – There are two phases to the behavioral response to injection of formalin. After an initial vigourous response, a period of reduced pain occurs 10 to 15 min after formalin, followed by reemergence of pain-related behaviors. These phases are believed to represent acute chemical stimulation of afferent neurons followed by injury-related inflammatory pain. Pentobarbital (10, 15, or 25 mg/kg), diazepam (0.5, 1.5, or 5.0 mg/kg), or ethanol (0.5, 1.0, or 1.5 g/kg) attenuated the diminution of pain between the two phases, so that pain was continuous throughout 60 min of testing, but had no effect on pain scores during the peaks of either phase. The effects of pentobarbital and diazepam were blocked by picrotoxin (2.5 mg/kg), which itself had no effect. Ro 15-1788 also blocked the effect of diazepam. Picrotoxin did not effectively antagonize the effect of ethanol. A high dose of picrotoxin (5.0 mg/kg) caused seizures in some rats and also eliminated the interphase depression of pain. The results suggest that the biphasic time course of formalin pain is produced by a central antinociceptive mechanism that is inhibited by $GABA_A$ receptors.

GABA receptors Pain Hyperalgesia Formalin Benzodiazepine Ethanol Pentobarbital Autoanalgesia Picrotoxin Ro 15-1788

THE formalin test (8) is an animal model of pain associated with tissue injury and inflammation that is sensitive to a variety of opioid and nonopioid analgesics (14,39). However, few known nonanalgesic drugs have been examined in this test. The sedative-hypnotic agents form a class of nonanalgesic drugs that are particularly significant for pain testing because they are widely used clinically, sometimes in conjunction with analgesics (20), and because their sedative actions may interfere with behaviors used to indicate pain in animal tests. Barbiturates and benzodiazepines have been reported to produce hyperalgesia (2-4,9), analgesia (2,17,19,26,28), to antagonize opioid analgesia (1,7,11,19,24,31,41), and, occasionally, to potentiate opioid analgesia (11). In the formalin test, diazepam (0.2 or 1 mg/kg) reduced the maximum effect of morphine, but there was no effect on pain in the absence of morphine (1), although the complete time course of formalin-induced pain was not examined in this study.

To investigate the effects of sedative-hypnotic agents on pain in the formalin test, the present study examined the time course of the formalin-induced pain response following injections of subanesthetic doses of pentobarbital, intoxicating doses of ethanol, and diazepam at higher doses than those previously tested. Since the sedative effects of pentobarbital and benzodiazepines are believed to be mediated by the GABA receptor-regulated chloride channel ($GABA_A$ receptor) (30) and ethanol has also been suggested to act through this chloride channel (37,38), the effects of the GABA antagonist, picrotoxin (40), and the specific benzodiazepine antagonist RO 15-1788 (18) were also examined.

METHOD

Procedure

Male Long-Evans rats, 250-350 g, were obtained from Charles River, Que., Ltd. and housed in cages of three to four with ad lib food and water. Groups of six were assigned to be

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tested to each drug or drug combination. The formalin test was conducted in a Plexiglas viewing chamber with a mirror mounted beneath the floor (14). They were exposed to this apparatus and test room for two sessions of 20 min on 2 separate days before testing. On the test day, each rat was injected with the appropriate drug or drugs (see below) and, after allowing time for absorption, 0.05 ml of 2.5% formalin (or 1% formalin in one experiment) was injected SC into the plantar surface of one hind paw. The rat was then placed in the viewing chamber and pain was rated for 60 min. The experimenter used a computer to record the amounts of time rats walked or stood firmly on the injected paw (pain = 0), partially elevated or favoured the paw (pain = 1), elevated the paw without contact with the floor (pain = 2), or licked, chewed, or shook the paw (pain = 3). The mean pain score was calculated at 5-min intervals by weighting the time a rat spent in each category of behavior by the pain score for that category, and dividing by the total time.

Drugs

All drugs except ethanol were injected in a volume of 1 ml/kg. Pentobarbital was diluted with saline (10, 15, or 25 mg/ml) and injected 10 min before formalin. Diazepam (Valium, Roche, 5 mg/ml) was diluted with 50 : 50 (v/v) propylene glycol : water solution to 0.5, 1.5, or 5.0 mg/ml and injected 30 min before formalin. RO 15-1788 (15 mg/ml) was suspended in the diazepam vehicle and given at the same time as diazepam. Ethanol (100%) was mixed with saline to concentrations of 0.5, 1.0, or 1.5 g/ml and administered 6 ml/kg, 5 min before formalin. Picrotoxin was acidified with 60 μl 0.1 molar HCl and 940 μl distilled water was added to dissolve the drug. Picrotoxin was injected 30 min before formalin. Pentobarbital, ethanol, their vehicles, and picrotoxin were injected IP. Diazepam, its vehicle, and RO 15-1788 were injected SC.

Data Analysis

Effects on the time course of the pain response to formalin were analyzed using groups by repeated measures ANOVA. In the presence of a statistically significant time by group interaction, the nature of the interaction was explored using simple effects analysis. Drug interactions were examined by comparing mean scores for the relevant portion of the time-effect relation using one-way ANOVA was followed by Tukey's protected *t*s.

RESULTS

Pentobarbital

The vehicle control group time curve in Fig. 1 illustrates the characteristic biphasic time course of the pain response to intraplantar formalin. The animals respond vigorously immediately after the injection, but after 5 to 10 min the initial response fades and there is a latency of 5 to 15 min before a second phase of pain appears. Pentobarbital markedly altered this time course (time by group interaction; $p < 0.015$). Specifically, the frequency of responses indicative of pain during the interphase period 10 and 15 min after formalin, when pain scores in control rats were low, were increased ($p < 0.005$). However, there were no differences between the groups for the remainder of the test.

Pain scores in the formalin test rarely exceed 2.5, and it was possible that the lack of effect of pentobarbital except during the interphase period was due to a behavioral ceiling that limited the rat's ability to express higher levels of pain. We therefore tested the effect of 15 mg/kg pentobarbital with a lower dose of formalin (1%). It can be seen in Fig. 2 that 1% formalin elicited substantially lower pain scores. There was little pain in the first 5 min, after which the scores increased gradually over 15 to 20 min. Pentobarbital did not alter the time course of pain with the lower formalin dose.

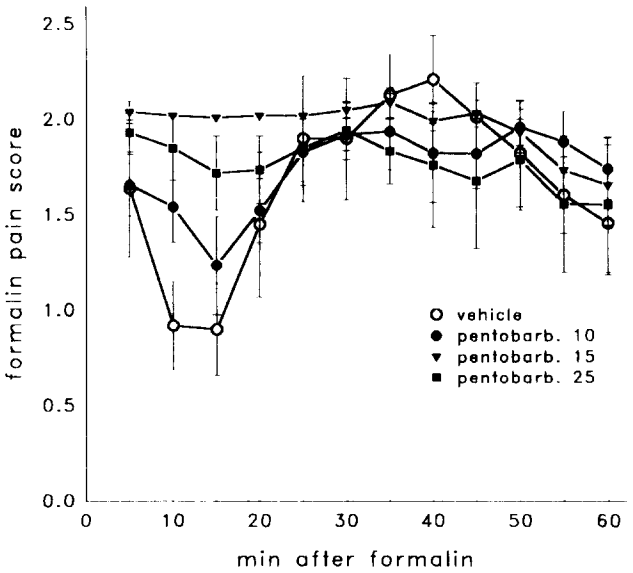

FIG. 1. Time course of the pain response following SC injection of 0.05 ml 2.5% formalin in separate groups of rats given 10, 15, or 25 mg/kg pentobarbital or the saline vehicle. Vertical bars indicate SEM.

Picrotoxin (2.5 mg/kg) did not alter the time course of formalin pain (data not shown; ANOVA: all *p*s > 0.05). A higher dose of picrotoxin (5 mg/kg) induced seizures in two out of six subjects and increased interphase pain responding, completely eliminating the period of reduced pain 10–15 min after formalin, even in subjects that did not exhibit seizures. This dose was therefore not tested further.

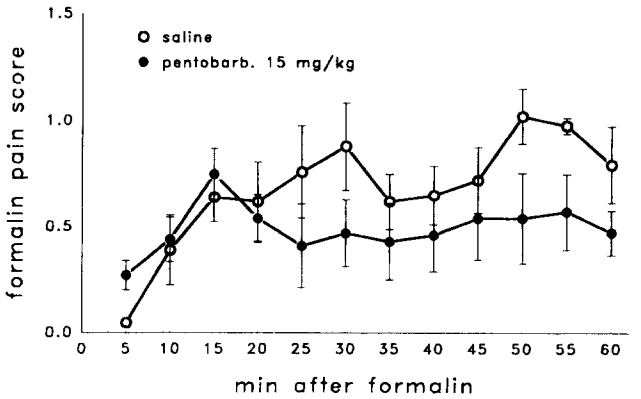

FIG. 2. Time course of the pain response following a SC injection of 1.0% formalin in groups of rats given saline or 15 mg/kg pentobarbital. Vertical bars indicate SEM.

Since effects of pentobarbital were restricted to the interphase period, drug interactions were examined using the mean of pain scores for the 10- and 15-min time bins. Figure 3 shows the mean pain scores for the interphase period for pentobarbital, picrotoxin, and the two agents combined. Pain scores were higher under all three doses of pentobarbital compared to controls treated with the saline vehicle ($p < 0.05$). Pentobarbital 15 mg/kg, but not 25 mg/kg, produced more hyperalgesia than 10 mg/kg. As indicated in the figure, picrotoxin did not significantly alter the pain response, but when combined with pentobarbital, picrotoxin attenuated the increased pain response produced by 15 mg/kg pentobarbital ($p < 0.01$).

Diazepam

As can be seen in Fig. 4, the effect of diazepam on the time-effect curves for formalin was very similar to the effect of pentobarbital (time by group interaction, $p < 0.0001$). The effect of diazepam was confined to increasing the pain scores during the periods 10, 15, and 20 min after formalin ($p < 0.05$). From a qualitative standpoint, the rats that received the highest dose of diazepam showed marked loss of motor tone to the extent that they were floppy when handled. Despite this, pain behaviors were unaffected.

Figure 5 shows the mean scores for the periods 10 and 15 min after formalin for diazepam, for RO 15-1788 alone, and for RO 15-1788 and picrotoxin in combination with diazepam. It is clear that the increase in pain scores produced by diazepam is dose dependent, reaching statistical significance for 1.5 and 5.0 mg/kg diazepam ($p < 0.01$). Like picrotoxin, RO 15-1788 by itself did not significantly alter the time course of formalin pain. Both RO 15-1788 ($p < 0.01$) and picrotoxin ($p < 0.01$) antagonized the pain-increasing effect of 1.5 mg/kg diazepam such that the two drug combinations were not different from vehicle-treated controls.

Alcohol

The effect of alcohol on the time course of formalin pain was not as marked as the effects of pentobarbital or diazepam (Fig. 6), but there was a highly significant time by group interaction ($p < 0.0001$) and pain scores were significantly increased 10, 15, and 20 min after formalin in alcohol-treated rats relative to controls ($p < 0.05$). Higher doses were not tested because at 1.5 g/kg, rats were sedated and ataxic for the entire period of testing. At the lower doses, rats showed a brief period of ataxia, but recovered after a few minutes. They appeared to groom themselves more than usual, but did not selectively groom the formalin-injected paw. Picrotoxin appeared to reduce the effect of 1.0 mg/kg alcohol during the interphase period, but the change did not reach significance (Fig. 7).

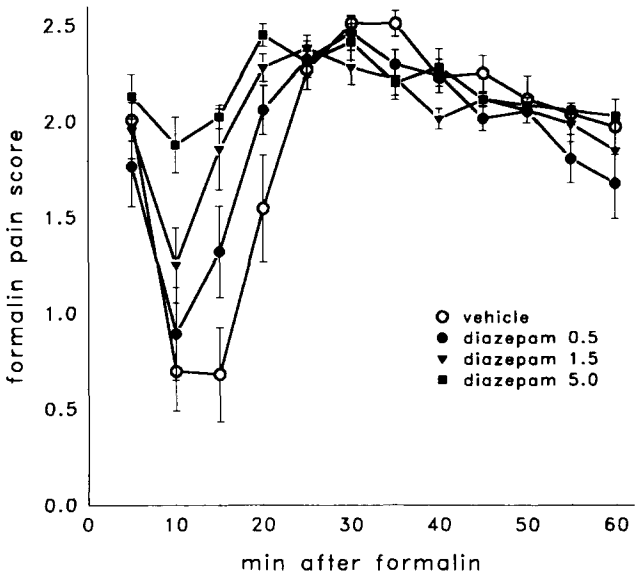

FIG. 4. Time course of the pain response following SC injection of 0.05 ml 2.5% formalin in separate groups of rats given saline or 0.5, 1.5, or 5.0 mg/kg diazepam. Vertical bars indicate SEM.

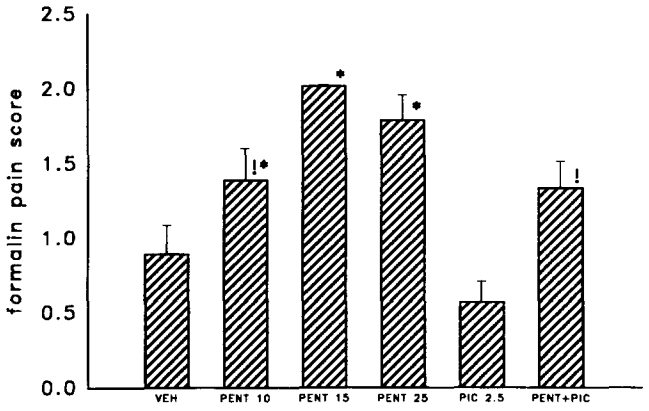

FIG. 3. Mean ± SEM pain score for the 10- and 15-min time bins after SC injection of 0.05 ml 2.5% formalin for groups of rats given vehicle (VEH), or pentobarbital (PENT) 10, 15, or 25 mg/kg (data replotted from Fig. 1), or 2.5 mg/kg picrotoxin (PIC) alone or in combination with 15 mg/kg pentobarbital. *Significantly different from vehicle ($p < 0.05$); !significantly different from pentobarbital 15 mg/kg ($p < 0.05$).

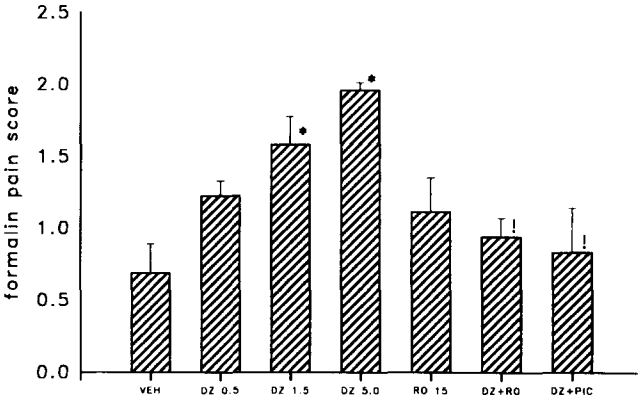

FIG. 5. Mean ± SEM pain scores for the period 5 to 15 min after SC injection of 0.05 ml 2.5% formalin for groups of rats given vehicle (VEH), diazepam (DZ) 0.5, 1.5, or 5.0 mg/kg (data replotted from Fig. 4), RO 15-1788 (RO) 15 mg/kg, RO 15-1788, or 2.5 mg/kg picrotoxin (PIC) in combination with 15 mg/kg diazepam. *Significantly different from vehicle ($p < 0.05$); †significantly different from diazepam 1.5 mg/kg ($p < 0.05$).

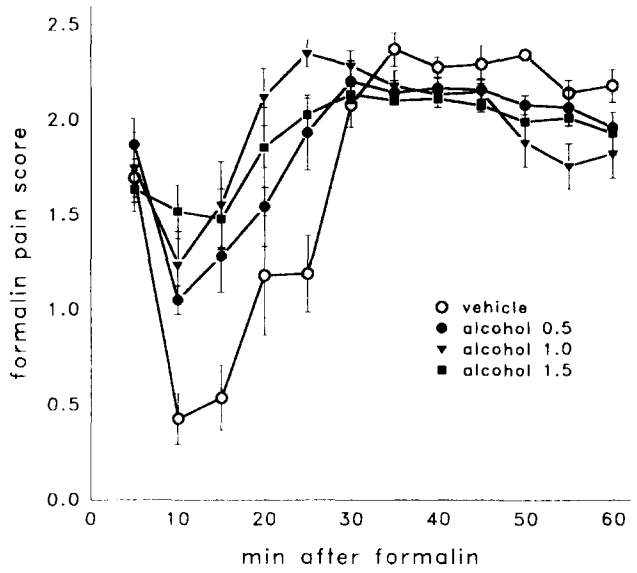

FIG. 6. Time course of the pain response following SC injection of 0.05 ml 2.5% formalin in separate groups of rats given vehicle or 0.5, 1.0, or 1.5 g/kg ethyl alcohol. Vertical bars indicate SEM.

Qualitative Changes in Pain Behavior

In vehicle control rats, paw lifting and shaking were high during the first 5 min after formalin, fell off during the interphase period, and rose to a peak in the second phase of pain. Paw favoring, which appears as limping when the animal is walking, was moderately frequent in the first 5 min, increased during the interphase period, fell during the onset of the second phase, and increased again during the decline of the second phase of pain. The sedative-hypnotic agents increased the frequency of paw lifting relative to other categories of behavior during the interphase period.

DISCUSSION

Our results show that pentobarbital, diazepam, and alcohol can increase the responsiveness to pain in the formalin test. The increased response to formalin was restricted to the period 10 to 20 min after formalin, when pain responding elicited by the formalin injection declines before increasing again in the second phase. In fact, pentobarbital, diazepam, and alcohol all eliminated the interphase decline in pain responding at the highest doses tested. These data are generally consistent with the clinical dictum that sedative-hypnotic agents cannot be used to relieve pain, and that hyperalgesia may be observed (32).

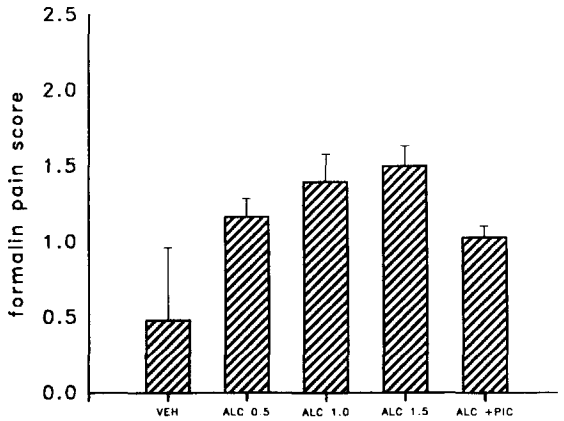

FIG. 7. Mean ± SEM pain scores for the period 5 to 15 min after SC injection of 0.05 ml 2.5% formalin for groups of rats given vehicle (VEH), alcohol (ALC) 0.5, 1.0, or 1.5 g/kg (data replotted from Fig. 6), or 2.5 mg/kg picrotoxin (PIC) in combination with 1.0 g/kg alcohol.

The results demonstrate that the formalin test shows good selectivity in both the early and late phase of pain: drugs that produced sedation and motor effects failed to alter pain scores at two doses of formalin, whereas known analgesics do attenuate the response (14,27). This was particularly apparent in the rats treated with 1.5 g/kg alcohol, which continued to exhibit formalin-induced pain responses despite obvious ataxia.

The data also suggest that the enhancement of pain responding during the interphase period is mediated by $GABA_A$ receptors, at least for pentobarbital and diazepam, for the following reasons. The hyperalgesic effects of pentobarbital and diazepam were blocked by picrotoxin, which opposes the effects of GABA and pentobarbital on the GABA-modulated chloride channel (40). The effect of GABA on the chloride channel is also modulated by a benzodiazepine receptor site (30), and the hyperalgesic effect of diazepam was blocked by the specific benzodiazepine antagonist RO 15-1788. Alcohol, which stimulates GABA-modulated chloride transport (37), also enhanced pain responding in the interphase period, though the effect was not significantly antagonized by picrotoxin.

A relatively high dose of picrotoxin (5 mg/kg) itself eliminated the interphase decline in pain responding. As the dose was high enough to elicit frank seizures in some animals, the effect on pain is difficult to interpret. On the other hand, the finding is compatible with the suggestion that there are antagonistic GABAergic pain-modulating systems (2,12). One of these acts to inhibit nociceptive input, and to potentiate the inhibitory action of morphine in the spinal cord (2,31). There are also GABA receptors in the periaqueductal grey, which are thought to tonically inhibit neurons projecting to the caudal brain stem. It has been proposed that opioids act by inhibiting these GABA interneurons, thus activating descending monoaminergic antinociceptive mechanisms (24,31, 34). In a complex system with multiple inhibitory controls, the effects of agonists and antagonists may be unpredictable, varying in relation to the dose and the spontaneous activity of the various components. It seems reasonable to suppose that GABA agonists might act in the periaqueductal grey to produce hyperalgesia by suppressing activation of the descending antinociceptive mechanisms. It is possible, on the other hand, that at high doses GABA antagonists could produce hyperalgesia by blocking GABA-mediated inhibition in the spinal cord, or that seizures disrupt the activity of forebrain antinociceptive mechanisms.

The effect of the GABA-modulating agents on the time course of formalin-induced pain behaviors raises some questions about the interpretation of the biphasic time course of pain in the formalin test. A few minutes after the initial vigorous response evoked by formalin injection, pain responding diminishes, then rises again to reach a maximum around 30 to 40 min after formalin. It has been assumed that the initial response is due to direct stimulation of nociceptors by formalin or tissue damage, while the second phase is due to subsequent inflammation (36). One explanation is that GABA agonists enhance or accelerate the development of the inflam-

matory phase of formalin pain. Alternatively, the pain generating mechanisms may be unchanged, but the GABA agonists unmask pain that is suppressed by some inhibitory mechanism.

Enhancement of inflammatory pain by GABA agonists is very unlikely. There are GABA receptors on Aδ and C fibres (6) that might modulate pain transmission to the dorsal horn or alter the neurogenic component of inflammation through inhibition of substance P release (33). However, GABA produces presynaptic inhibition via these receptors (6). Drugs that increase GABA-mediated inhibition, such as alcohol, pentobarbital, and diazepam, enhance presynaptic inhibition (5,33), and would thus be expected to reduce pain transmission or neurogenic inflammation. Moreover, if inflammation were increased one would expect to see an increase in pain over the time course of the inflammatory process. However, neither the putative GABA agonists nor their antagonists had any effect on pain except during the interphase period. Even with 1% formalin, which produces a submaximal pain response, there was no effect of pentobarbital on what is assumed to be the phase of inflammatory pain.

Several lines of evidence favour the notion that these drugs may unmask pain that is suppressed by a central antinociceptive mechanism. Firstly, one other treatment that eliminates the interphase diminution of pain, without altering the second phase, is transection of the neuraxis at the mesencephalic–diencephalic junction (25). The suggestion that the interphase diminution of pain might be due to an antinociceptive influence that originates rostral to the transection (25) is supported by the finding that the biphasic character of the formalin pain response is not present in the neonatal rat, but appears between days 10 and 15 (15), when descending inhibitory controls become functionally mature (13). Secondly, painful stimuli activate descending inhibitory controls (22) and attenuate behavioral reactions to pain (autoanalgesia) (10,16), and some forms of autoanalgesia are blocked by sedative antianxiety drugs such as pentobarbital and the benzodiazepines (23,35). At present, it is not possible to identify the type of autoanalgesia that might be involved, except that it is probably a non-opioid autoanalgesia, because the opioid antagonist, naloxone, does not alter the biphasic time course of formalin pain (21,29).

Finally, it should be noted that the hypothesis that the temporary diminution in formalin pain may be due to autoanalgesia does not contradict the view that there are two phases of formalin-induced pain. Rather, the data presented here suggest that the behavioral time course of formalin pain may not be a reliable indicator of the transition from pain induced by the injury to pain arising from inflammation. In fact, it appears that this transition may occur without a substantial diminution of the overall pain stimulus.

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