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Morphine Analgesia and Tolerance in the Tail-Flick and Formalin Tests: Dose-Response Relationships

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ABBOTT, F. V., R. MELZACK AND B. F. LEBER. *Morphine analgesia and tolerance in the tail-flick and formalin tests: Dose-response relationships.* PHARMAC. BIOCHEM. BEHAV. 17(6) 1213–1219, 1982.—The dose-response relationships for morphine analgesia were studied in morphine-tolerant and non-tolerant rats using two pain tests: the tail-flick test which measures the threshold for an escape response, and the formalin test which assesses the behavioral response to continuous pain generated in injured tissue. The effects of prior experience with both pain tests on tolerance were also examined. In the formalin test, effective analgesia was obtained in non-tolerant rats at doses that produce minimal depression of locomotor behavior. Morphine tolerance was produced by 20 daily injections of morphine with increments that reached 16 mg/kg, a dose over the LD_{100} for barrier sustained Long Evans rats. This dose regimen produced a 1.8-fold increase in the ED_{50} in the tail-flick test and a 2.7-fold increase in the formalin test. Daily experience of the pain test, as well as the morphine regimen produced a 4.8-fold increase in the ED_{50} in the tail-flick test but did not affect the potency of morphine in the formalin test. The magnitude of tolerance in the absence of daily behavioral testing is consistent with recent clinical reports that little tolerance occurs after prolonged administration of morphine in cancer patients and that tolerance is not an important consideration in the management of pain.

Morphine Tolerance Pain Opiates Environmental effects Tail-flick test Formalin test

THE formalin test [13] is different from most models of pain since it assesses the way a rat responds to moderate, continuous pain generated by injured tissue instead of measuring the threshold for an escape response such as the tail-flick. It is not surprising, therefore, that the neural mechanisms that underlie morphine analgesia in the formalin test are markedly different from those in the tail-flick test. For example, antiserotonergic drugs that attenuate the effects of morphine in the tail-flick test potentiate low doses of morphine in the formalin test [12]. Moreover, the blockade of morphine analgesia by antiserotonergic agents in the tail-flick and other withdrawal-reflex pain tests has been shown to be due to interruption of a bulbospinal 5HT pathway that originates in the nucleus raphe magnus (see [9,25] for reviews), but lesions of this nucleus do not have any effect on morphine analgesia in the formalin test [1,2].

Another point of difference between the conventional pain tests and the formalin test is in the development of tolerance—that is, the decrements in analgesia following repeated administration of morphine. Abbott *et al.* [3] and Abbott [4] failed to detect a significant decrease in analgesia in the formalin test after 20 days of morphine using doses ranging from 1.87 to 30 mg/kg. Instead, doses ranging from 3.75 to 30 mg/kg became equipotent after the rats became tolerant to the locomotor depressant effect of morphine. In the same dose range, marked tolerance occurred in the tail-flick test, but the decrease in potency was much less than that reported by Mucha *et al.* [29]. This may have been due to the fact that Mucha *et al.* tested rats every day during the development of tolerance, a procedure which would be expected to maximize conditioned tolerance [8,32]. The design of our experiment did not allow us to separate the effects of repeated testing from pharmacological tolerance. Another problem with our earlier studies was that the formalin test was much more sensitive to morphine than we expected and the dose-response function was incomplete.

Because the differences between the formalin and the tail-flick tests may reflect differences in both the nature of the pain involved and the mechanisms of analgesia, it is necessary to obtain more information on the pharmacology of morphine in the formalin test. The present experiment was designed to obtain quantitative estimates of the decrements in analgesia in the tail-flick and formalin tests. To this end all rats received the same high dose of morphine and were subsequently tested at a series of doses. To assess possible effects of exposure to the test procedures on these decrements, separate groups of rats were given either morphine or saline in each test environment.

METHOD

Subjects

The subjects were 190 Long Evans hooded rats weighing 200–250 g at the beginning of the experiment. They were

housed in group cages of 3 or 4 in the colony room. Food and water were available ad lib.

Morphine Administration

The morphine sulphate was dissolved in normal saline and injected SC, 1 ml/kg of body weight. Tolerance was produced by injecting the rats with 8 mg/kg of morphine daily for 8 days followed by 16 mg/kg for 12 days. Mortality from this regimen was about 25%. Test doses ranging from 0.5 to 32 mg/kg were used. All doses are expressed as the salt.

Tail-Flick Test

The tail-flick test was carried out with the rat restrained in a narrow cone made of fine-gauge wire screen. A wire bar inserted at the open end prevented the rat from backing out but allowed the rat's tail to hang down. A cloth was then draped over the restrainer. This apparatus appeared to be less stressful than other standard restrainers because defecation and struggling were rare.

The test was performed by quickly immersing the distal 5 cm of the tail in a beaker of water and timing the latency until the rat curled its tail out of the water. A tissue bath regulator was used to maintain the water at 55°±0.2°C. If no response occurred in 10 sec, the trial was terminated. The tail was dried with a towel immediately to prevent redness and swelling. With this procedure, we have found that an individual rat's response time is consistent in successive tests 10 min or several days apart.

The tail-flick latency was measured before and 15, 30 and 60 min after the morphine injection. This provides 3 measures spanning the period of time after drug injection that pain was rated in the rats that received the formalin test.

Formalin Test

The formalin test was carried out in a 30×30×30 cm clear Plexiglas box. A mirror was mounted at a 45° angle below the transparent floor to allow an unobstructed view of the rat's paws. To assess the effects of morphine on locomotor activity during the pain test, the floor of the box was pivoted so that the rat's movement across the floor produced closure of a switch. At right angles to the axis of tilt, a photocell was mounted so that interruption of the light beam also caused closure of a switch. Both switches were connected to a single counter that provided a total measure of activity.

The procedure was as follows: the rat was weighed, taken to the experimental room, injected with the appropriate dose of morphine or with saline, and placed in the test chamber for 10 min. The rat was then removed from the chamber and the formalin test began.

The formalin test consisted of injecting 0.03 ml of 2.5% formol saline subcutaneously into the dorsal surface of the rat's forepaw. This amount of formalin, which is less than that used by Dubuisson and Dennis [13], produces less tissue damage without altering the sequence and duration of pain responses [3,4]. Immediately after the formalin injection, an observer began recording the amount of time the injected paw was held in an elevated position so that the ventral surface of the paw did not touch the floor (Fig. 1). The pain score was derived by calculating the percentage of time the injected paw was elevated in each 10 min time block for 40 min.

During the period of the pain test, locomotor activity was recorded. This is important because morphine interacts with exposure to different environments to produce different activity levels. If the levels of analgesia were to be correlated with activity levels rather than the dose of morphine, the test would clearly be invalid as a measure of pain.

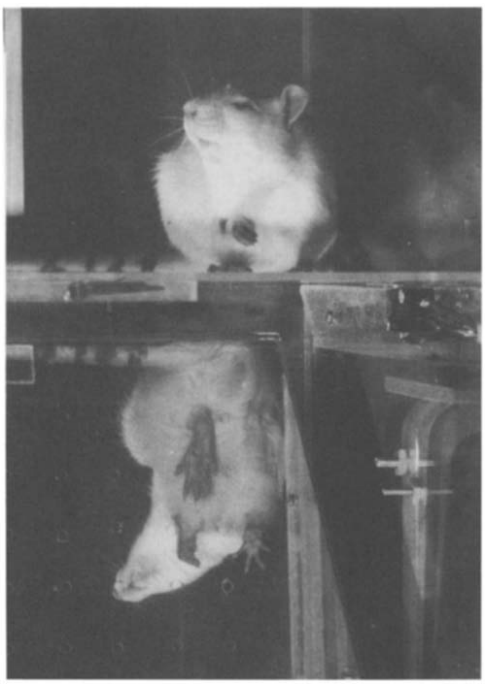

FIG. 1. Typical pain behavior of a rat following the formalin injection into a forepaw.

Experimental Design

An independent-group design was used so that each rat was only tested at a single dose of morphine on one of the tests. In the tail-flick test, groups of 4 or 5 rats were used at each dose level. In the formalin test group size varied from 5 to 7.

Table 1 presents the prior test history, drug experience, type of pain test, and the test doses of morphine for each group of rats. The test-naive rats received one of the pain tests for the first time on the test day using one of the doses of morphine indicated in the table. Tail-flick experienced rats were injected with either saline or the chronic morphine regimen each day for 20 days and received exposure to the complete tail-flick test procedure; on the test day they were tested with the doses indicated in the table. The hypertonic-saline experienced group received a modified form of the formalin test, using hypertonic saline, because the tissue damage caused by the formalin test precludes repeated testing. These rats were given their daily saline or morphine injection and placed in the formalin test chamber. Ten min later, 0.05 ml of 4.5% hypertonic saline was injected SC into a forepaw and the rat was replaced in the test chamber for 30 min. The left and right paws were injected on alternate days. The hypertonic saline injection causes a pain response that lasts 3–5 min. While this is considerably less than the pain produced by a formalin injection, it exposed the rats to the test environment, procedure and some pain without destroying their paw and would be expected to produce at least some situation-specific tolerance. The group that received daily saline instead of morphine served as a control for the

TABLE 1
EXPERIMENTAL DESIGN

Prior test history	Drug experience	Type of pain test	Test Doses (mg/kg)
Test naive	No injection	Tail-flick	1; 2; 4; 8; 16
		Formalin	0; 0.5; 1; 2; 4; 8
	Morphine injected	Tail-flick	1; 2; 4; 8; 16; 32
		Formalin	0; 0.5; 1; 2; 4; 8; 16; 20
Tail-flick experienced	Saline injected	Tail-flick	1; 4; 16
	Morphine injected	Tail-flick	1; 4; 16; 32
Hypertonic saline experienced	Saline injected	Formalin	2; 4
	Morphine injected	Formalin	2; 4

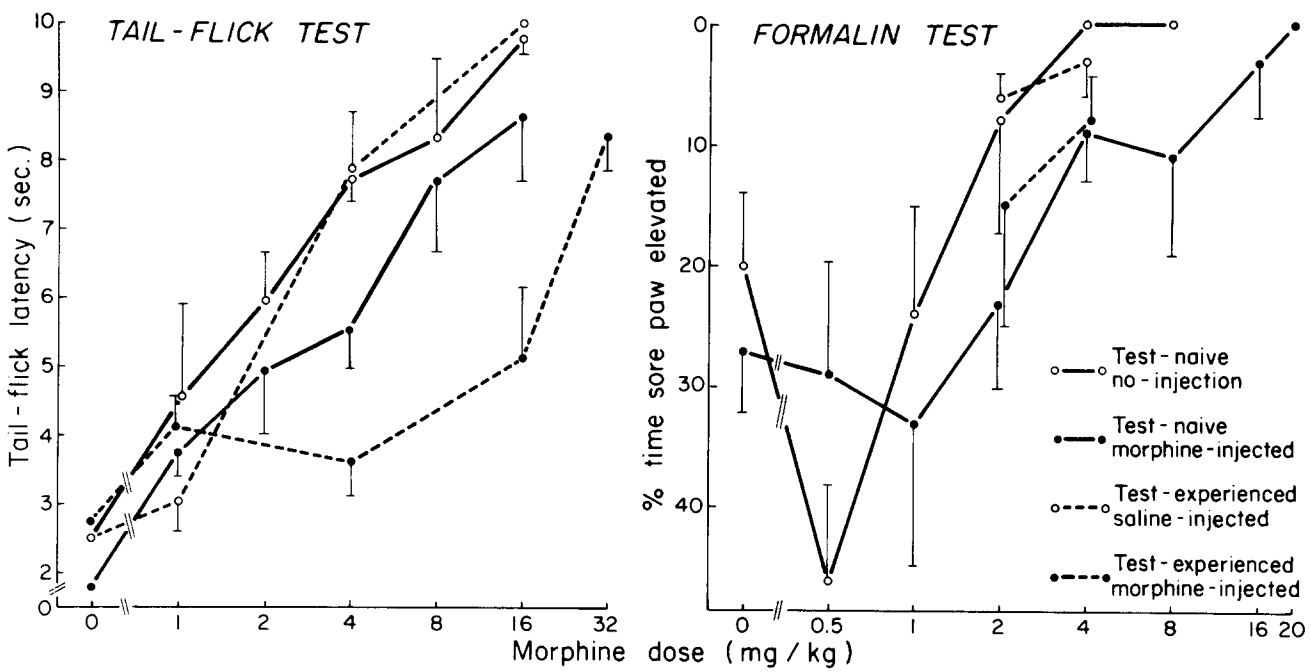

FIG. 2. Dose-response functions for morphine in the tail-flick and formalin tests. The baseline pain levels in the tail-flick test are preinjection scores while in the formalin test they are separate groups of rats.

possible effects of tissue damage produced by the hypertonic saline injections since they were tested after their first dose of morphine.

The data were analyzed using ANOVA's to test for differences between groups when morphine doses overlapped; for example, between test-naive rats with and without morphine pretreatment. In the formalin test, variances tend to be non-homogeneous and correlated with the means [4]. Therefore, the F's reported for the formalin test are for square-root transformed pain scores.

RESULTS

Figure 2 shows the baseline pain scores and the morphine dose-response functions for test-naive and test-experienced rats with and without 20 days of morphine pretreatment. The

TABLE 2

ED_{50}'s FOR TAIL-FLICK AND FORMALIN TESTS

Prior test history	Drug experience	Type of pain test	Test Doses (mg/kg)	ED_{50} (mg/kg) Peak Effect	ED_{50} (mg/kg) Mean Over 1 Hour
Test naive	No Injection	Tail-flick	1,2,4,8,16	3.62	4.09
		Formalin	0.5,1,2,4	1.06	1.96
	Morphine injected	Tail-Flick	1,2,4,8,16	6.50	8.17
		Formalin	1,2,4,8,16	2.93	4.16
Test experienced	Saline injected	Tail-flick	1,4,16	4.54	3.97
	Morphine injected	Tail-flick	4,16,32	17.51	27.70

data shown for the tail-flick test are the latency for tail withdrawal tested 30 min after morphine administration. For the formalin test, the mean pain levels for the 10 min period 30–40 min after morphine administration are shown.

The baseline tail-flick latencies are the means for the pre-morphine tail-flick test on the test day for the various groups which were then subdivided according to morphine test dose. The baseline scores for the test-naive morphine-injected group were significantly lower than those for the other 3 groups ($p<0.05$, Newman Keuls). Since there was no evidence of hyperalgesia in the group that was given daily morphine and test experience, it is possible that the effect is due to hyper-reactivity as a result of putting the rats into an unfamiliar environment at the time of day when they would normally get their daily morphine injection. This is supported by the fact that many of the rats responded vigorously and immediately (latencies less than 1 sec) to the water simply touching the tail.

The formalin test baselines scores are from 2 separate groups of test-naive rats with and without 20 days of morphine administration. The difference between the two groups was not significant at any time during the test, $F(1,12)=1.52$, $p>0.2$.

The solid lines in Fig. 2 show the dose-response functions for morphine in test-naive rats. In the tail-flick test, the dose-response function for morphine lies between 1 and 16 mg/kg in rats of the Long Evans hooded strain with no previous experience of morphine. It is noteworthy that most of the rats given 16 mg/kg for this test later died of respiratory failure. In the formalin test, the rising portion of the dose-response curve lies entirely between 0.5 and 4.0 mg/kg, a dose which does not produce significant depression of locomotion (activity measures are discussed below). It is interesting that at 0.5 mg/kg, high pain scores, suggesting hyperalgesia, are seen in the formalin test. This effect has been observed in other experiments (unpublished) but only in the present experiment has it reached statistical significance.

Pretreatment with morphine over a 20-day period produced a significant change in the dose-response curves (tail-flick, $F(1,29)=6.89$, $p<0.02$; formalin, $F(1,40)=10.66$, $p<0.01$. ED_{50}'s (Table 2) were calculated from regression analyses of the straight portions of the dose-response curves. To calculate the ED_{50}'s, a tail-flick latency of 7 sec and a formalin score of 10% of time with the paw elevated were used as the 50% "analgesia" values. As illustrated in Fig. 2 (solid lines), for both pain tests this change shifted the dose-response curves for test-naive rats to the right so that approximately double the dose of morphine was required to produce an equi-analgesic effect. The ED_{50} ratios calculated from Table 2 by dividing the ED_{50}'s for the untreated rats are, in fact, 1.80 for the tail-flick test and 2.75 for the formalin test.

Figure 2 (dotted lines) shows the effects of exposure to the pain tests with and without morphine for 20 days. Twenty days of saline injections plus daily test experience did not alter the effects of the first morphine dose (tail-flick, $F(1,21)=1.67$, $p>0.2$; formalin test $F(1,35)=0.01$, $p>0.09$). Exposure to the test plus pretreatment with morphine produced the only striking difference between the two tests. As indicated in Table 2, in the tail-flick test this increased the ED_{50} from 3.62 to 17.51 mg/kg, a 4.84-fold increase. Pretreatment with morphine alone produced only a 1.80-fold increase in the ED_{50}. In contrast, in the formalin test there was no further decrease in the effectiveness despite considerable fibrosis of the forepaws as a result of injecting hypertonic saline daily, $F(1,35)=0.23$, $p>0.6$. The lack of effect of exposure to the test is unlikely to be due to diminished pain sensitivity produced by the hypertonic saline because the degree of analgesia for the test-experienced, saline-injected rats was the same as that seen in test-naive rats receiving their first dose of morphine. It was not possible to calculate the ED_{50}'s for these formalin groups.

Preatment with morphine significantly altered the time-course of drug action so that the onset of the drug effects was slower and the effects dissipated more quickly, particularly for the lower doses (time × dose × tolerance interactions: tail flick, $F(8,58)=2.49$, $p<0.05$; formalin, $F(12,112)=2.01$, $p<0.05$). It is interesting to note (Table 2), however, that when the data were averaged over 1 hr, the magnitude and patterns of tolerance for both pain tests were very similar to those shown in Fig. 2.

Figure 3 shows the mean activity scores for rats during the formalin test. It can be seen that naive rats with no prior

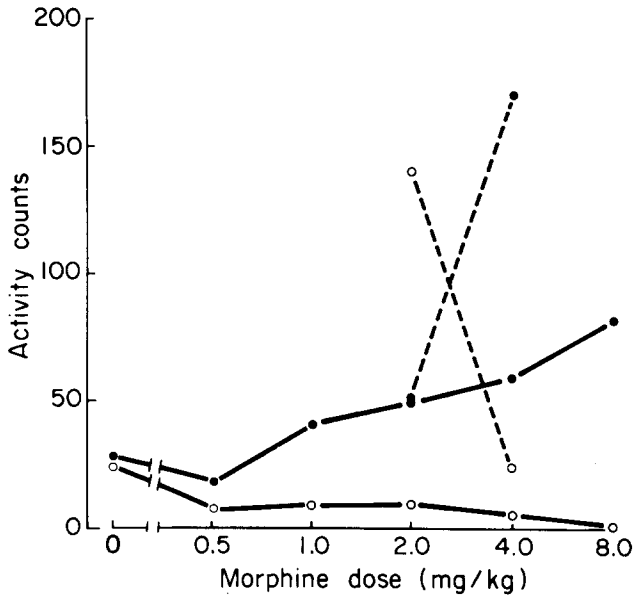

FIG. 3. The effects of morphine on locomotor activity recorded during the formalin test. The symbols are the same as those used in Fig. 2.

experience of morphine move around very little in the test chamber and show a decrease in activity at doses between 2 and 8 mg/kg. Pretreatment with morphine produced dose-dependent hyperactivity. In contrast, the test-experienced groups which received either morphine or saline each day show marked but opposite effects. The saline group was hyperactive at 2 mg/kg while at 4 mg/kg the depressant effect of morphine predominated. The morphine-treated rats showed more exaggerated dose-dependent hyperactivity than the rats that received morphine alone. These data are consistent with previous reports of the effects of morphine on activity [4,7]. The most important point in this context of this experiment is that while manipulation of the environment produces large changes in activity levels, the pain measure at a given dose of morphine is not altered.

DISCUSSION

Dose-Response Curves in Rats with no Previous Morphine

The dose-response curves for morphine naive rats in these experiments indicate that there is a progressive increase in analgesia as the morphine dose increases in the formalin test (Fig. 2). The slope is steep and the rising phase of the curve lies entirely between 0.5 and 4.0 mg/kg, below the dose at which marked depression of locomotion occurs (Fig. 3). In man, adequate analgesia without marked sedation is an important property of opiates. In contrast, in the tail-flick test, 16 mg/kg of morphine is required before all rats fail to remove their tails from the water bath within the 10 sec cut-off time. This cut-off time was chosen because experience has shown that, using a 55°C water bath, longer exposure leads to tissue damage as indicated by the fact that subsequent tail-flick latencies are shorter and there is erythema and swelling. Thus, a lethal dose of morphine was necessary to suppress a withdrawal reflex which would prevent tissue damage.

It is possible that these dose-relationships only indicate that the formalin test produces less pain than the tail-flick test. However, this relationship is consistent with clinical data. Ten mg of morphine produces adequate analgesia in 80% of post-operative patients, [15,21] but double this dose, 20 mg, is required to consistently raise the heat pain threshold in man [18,23].

The Effects of Chronic Morphine Adminiatration

In both the formalin and tail-flick tests, pretreatment with 20 injections of morphine building up to 16 mg/kg made it necessary to approximately double the doses to produce analgesia equivalent to that seen in untreated rats—that is, a 2-fold right shift of the dose-response curves (Fig. 2 and Table 1). This shift is much smaller than expected. Mucha *et al.* [30] found about a 10-fold shift at a comparable dose, 25 mg/kg IP., which is about equivalent to 14 mg/kg SC [19]. The fact that the shift is consistent in both tests suggests that it reflects a basic pharmacological change. It is also consistent with our previous failure to detect any tolerance in the formalin test [3,4] when the test dose of morphine was administered chronically. With effects of this magnitude, it is necessary to administer high doses and challenge with low doses to demonstrate tolerance.

If this 2-fold shift is accepted as the degree of pharmacological tolerance, then the surprising finding is that exposure to both morphine *and* the tail-flick test produced a 3-fold decrease in the analgesic effects over and above the 2-fold decrease produced by morphine administration alone. The difference between the test-experienced and test-naive morphine-tolerant rats suggests that at least two processes may be occurring during the development of tolerance. This is in agreement with the two-component tolerance curve that Mucha *et al.* [30] found in rats tested every day. The effect of exposure to the test environment on tolerance has been attributed to "conditioning" [33,36]. In this model it is proposed that the environment in which a drug is administered acquires the ability to trigger physiological and behavioral compensatory responses. The "conditioning" hypothesis is considerably weakened by the finding that test experience produces increased tolerance even when morphine is administered by a pellet implant which removes all drug administration cues [5,6]. Nevertheless the present experiment indicates the magnitude of environmental effects on tolerance is very large and the phenomenon certainly warrants further investigation. The fact that there was no effect of environment on tolerance in the formalin test indicates that it does not occur in all types of pain and may not normally be involved in the clinical use of morphine.

Tolerance to the analgesic effects of morphine and other narcotic drugs is a widely accepted phenomenon. However, there is little agreement in the clinical literature on its magnitude. For example, Houde *et al.* [16] report that it was necessary to approximately double the dose of morphine in 7 days to obtain an equi-analgesic dose in 7 cancer patients. On the other hand, Twycross [34,35] and Mount *et al.* [28] state that tolerance is not a practical problem in the management of pain in terminal cancer in about 85–90% of the cases. This is not due to the administration of other drugs in the Brompton mixture ([27]; T. Walsh, personal communication). In the remaining 10–15% of patients, pain is intractable even with large doses of narcotics [26]. Similarly, Isbell *et al.* [17] also report good pain control in surgical patients for periods of 3 to 5 weeks without large increases in the dose of narcotics. Narcotic addicts on methadone maintenance programs also do not require heroic doses of narcotics to control post-surgical pain [20,31].

On the other hand the experimental literature suggests

there is rapid tolerance to narcotic analgesia [8, 14, 24, 32]. For example, Mucha *et al.* [30] found that 24 days of 8 mg/kg/day of morphine produced about a 2-fold increase in the equi-analgesic dose in rats. During the tolerance development phase, the tail-flick latency showed a dramatic decrease and, depending on the dose, almost complete disappearance of the analgesic effects in 5 to 30 days. Experimental studies of tolerance in man have usually used the heat-pain threshold test in post-narcotic addict subjects, and the data support the findings in animals [17]. In fact, experimental pain models which failed to demonstrate tolerance to narcotic analgesics have been considered misleading and abandoned (e.g., the tail compression test in cats [14]).

The discrepancies in the literature appear to be due to the nature of the pain that is studied. Experimental models of pain commonly involve measuring the threshold at which pain (usually produced by intense heat) is reported or a withdrawal response occurs. This type of pain—"first pain" [29], "sharp pricking pain" [11], or "phasic pain" [12]—is well localized and serves to protect an organism against tissue damage. Moreover this type of pain is not very sensitive to narcotic analgesics [10, 15, 18, 23]. Thus, the tests most often used to study narcotic analgesia involve pain that is relatively insensitive to narcotics. In order to characterize pain and analgesia in animals, it may be necessary to also study the diffuse, poorly localized pain that follows tissue injury—"true pain" [11] or "tonic pain" [12].

Another issue that arises from our data is the possibility of a ceiling to the degree of analgesia obtainable by morphine in clinical practice. In rats that receive their first dose of morphine, pain scores in the formalin test always reach 0 at about 8 mg/kg. This pain score is, however, associated with marked locomotor depression which occurs at this dose (Fig. 3). At 8 and 16 mg/kg in morphine-tolerant rats when the depressant effects of morphine have abated, the dose-response curve appears to level off between 4 and 16 mg/kg. While the effect here is weak, it supports our previous finding that, when doses between 3.75 and 30 mg/kg are administered chronically, all doses become equipotent [4]. Lasagna and Beecher [22] noted that in clinical pain in man, there is little advantage to administering very large doses of opiates (i.e., greater than 20 mg morphine). The formalin test data presented here suggest that further study of this in a clinical setting is warranted.

In conclusion, the present experiments demonstrate that the formalin test is a valid, reliable model of pain in animals. The dose-response curve in non-tolerant rats indicates that good analgesia is produced at doses of morphine which produce minimal levels of locomotor depression. This is important because analgesia with minimal sedation is one of the cardinal properties of opiates. Furthermore, the degree of tolerance seen is consistent with clinical reports that tolerance is *not* an important consideration in the management of prolonged pain [26, 28, 34, 35]. The marked tolerance previously reported (eg., [14,32]) appears to be due to an interaction between the drug, the prior test history and the type of test used.

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