# The Increasing-Temperature Hot-Plate Test: An Improved Test of Nociception in Mice and Rats

ARNE TJØLSEN, JAN HENRIK ROSLAND, ODD-GEIR BERGE, AND KJELL HOLE

The increasing-temperature hot-plate test has several advantages compared to the conventional hot-plate test, but available equipment has been impractical and restricted with regard to stimulus control. We now describe an apparatus consisting of an aluminum plate that is heated and cooled by Peltier elements in contact with its lower surface. Several plates can be used simultaneously, individually controlled by electronic proportional feedback circuits. The set temperature of the feedback circuit is controlled by a computer program run on an IBM XT-compatible PC, so that a linear increase in temperature is achieved. Experiments were performed using rats and mice, with hindpaw licking as an end-point criterion. Experiments with various heating rates showed that 3.0°C/min is the lowest rate that can be applied without signs of stress in the animals. On the basis of the recorded data, nociceptive temperature thresholds were calculated to be approximately 44.5°C for both rats and mice. Inspection of the paws after analgesic treatment and exposure to different end-point temperatures suggested that a cutoff temperature of 50°C should be employed to minimize tissue damage. Testing at ambient temperatures of 18° and 28°C yielded similar results for rats, whereas mice responded at significantly higher plate temperatures in the colder environment. Dose-related antinociceptive effects were demonstrated for morphine and paracetamol in both species. The results confirm that the increasing-temperature hot-plate test is a valuable test of nociception, which is also suitable for demonstrating the antinociceptive effects of nonopioid analgesics. The test may also be used to estimate the nociceptive temperature threshold.

**Key Words:** Pain measurement; Hot-plate method; Paracetamol; Morphine; Rat; Mouse

## INTRODUCTION

The hot-plate test is commonly used to investigate nociception and analgesia in rodents. The standard method as described by Woolfe and MacDonald (Woolfe and MacDonald, 1944) and modified by Eddy and coworkers (Eddy, Touchberry, and Lieberman, 1950) records the latency for nociceptive responses of animals placed on a plate kept at constant temperature, usually about 55°C. Analgesic effects of morphine and other narcotic analgesics are easily identified using this test (Woolfe and MacDonald, 1944; Eddy et al., 1950). Analgesic effects of weak, nonnarcotic

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analgesics have been demonstrated at 50°C (Ankier, 1974), but the results show considerable variability, and it may therefore be difficult to obtain statistical significance (Taber, 1974).

In a recent modification of the test, animals are placed on a plate at a temperature below the nociceptive threshold and the temperature is increased at a constant rate (Ögren and Berge, 1984). This test has been found to be sensitive to weak analgesics, such as acetylsalicylic acid and paracetamol, in mice and paracetamol in rats (Hunskaar et al. 1986a and 1986b).

The apparatus previously described for the increasing-temperature hot-plate test consists of a conventional hot plate that shows a linear increase of temperature when the heating is turned to maximum. The conventional hot plate is commercially available. However, it has several disadvantages when used in the increasing-temperature hot-plate test. The rate of temperature increase cannot be controlled or adjusted, and the maximum rate of increase is 2°–3°C/min. Hence, it is impossible to do parametrical studies of the test. Furthermore, cooling of the conventional hot plate is time-consuming. The test equipment described in the present study has several advantages. The apparatus allows different rates of heating. We have therefore investigated the effect of this parameter on the response temperature. It is convenient; active cooling starts automatically when the end-point response is registered, and the time interval between tests is hereby considerably shortened.

## MATERIALS AND METHODS

### Animals

Male Sprague–Dawley rats (Mol:SPRD, Møllegaard, Denmark), weighing 290–350 g, and male NMRI mice (BOM:NMRI, Bom-mice, Ry, Denmark), weighing 25–30 g, were used. The animals were housed at 21°–24°C and the rats and mice (three and 15–16 to a cage, respectively, with free access to food and water) were kept on a 12:12 hr light/dark cycle with lights on at 7:00 AM. The observer was unaware of the drug treatment in each experiment. The animals were adapted to the cold plate for 1 min on the day before testing.

### Drugs

Morphine hydrochloride (NAF-laboratoriene a/s, Norway) was dissolved in 0.9% NaCl, and was injected subcutaneously in the neck in a volume of 5 ml/kg. Paracetamol (Svaneapoteket, Bergen) was dissolved in 6.25% propanediol in 0.9% NaCl, and was injected intraperitoneally in a volume of 15 ml/kg. Control animals were injected with the same volume of the appropriate vehicle.

### Increasing-Temperature Hot-Plate Apparatus

The hot plate measured 12 × 27 cm, was made of aluminum, and was enclosed by an unlidded perspex box, 30 cm high. Peltier elements fixed to the lower surface of the plate heated or cooled the plate depending on the direction of the applied current. Several hot plates can be operated simultaneously; in the experiments described here, two plates were used. The temperatures of the plates were indi-

vidually controlled by electronic proportional feedback circuits connected to a thermistor embedded centrally in the plate. The set temperature of the feedback circuit was controlled by a Basic computer program run on an IBM-XT-compatible Personal Computer via an IBM PC Data Acquisition and Control Adapter. The actual temperatures of the plates were monitored and displayed on the computer screen during experiments. Calibration was done by means of small thermocouple probes that were kept in thermal contact with the plate surface by means of heat-conducting paste. The readings from the thermocouple probes were themselves calibrated against a standard calibration mercury thermometer (±0.01°C). The surface temperatures were found to be lower close to the edge of the plates, with differences of up to about 1°C. Except for the peripheral 2 cm of the plates, the surface temperature was constant within 0.3°C.

The starting temperature was 42.0°C. In the experiment with different heating rates, the rate of heating was either 1.0, 2.0, 3.0, 4.5, or 6.0°C/min. In all the subsequent experiments the heating rate was 3.0°C/min. The nociceptive responses were recorded by the experimenter via the computer keyboard, and the response temperatures were recorded by means of the computer. Cooling of the plates started automatically when the final response was recorded. The rate of cooling was rapid, so that tests could be conducted every 5 min with a heating rate of 3.0°C/min.

### Testing Procedure

The animals were brought to the test room approximately 2 hr (rats) or 18 hr (mice) before experiments. The mice were placed in individual cages 2 hr before testing, whereas the rats were kept in their home cages. Testing took place between 10.00 and 15.00 hr and the ambient temperature during testing was between 21° and 24°C, except when the effect of different ambient temperatures was studied. For testing, the animals were gently placed on the hot plate at the starting temperature. The increase of the temperature started immediately at the predetermined rate. Licking of one of the hind paws was used as the nociceptive response.

### Statistics

Analysis of variance (ANOVA) (CSS statistical package) was used to evaluate drug effects. Student's *t* test was applied when the comparison was restricted to two means. Significance was accepted at the 5% level.

## RESULTS

### Effect of Different Heating Rates

Groups of untreated animals were tested at different heating rates; 1.0, 2.0, 3.0, 4.5, and 6.0°C/min. The temperature of the plates when the first and second lick or licking period occurred was recorded separately, and the response temperatures for the various heating rates are shown in Figure 1. The response temperatures increased with increased heating rates for both rats and mice.

At a rate of 1.0°C/min, and to a considerably less extent at 2.0°C/min, it was ob-

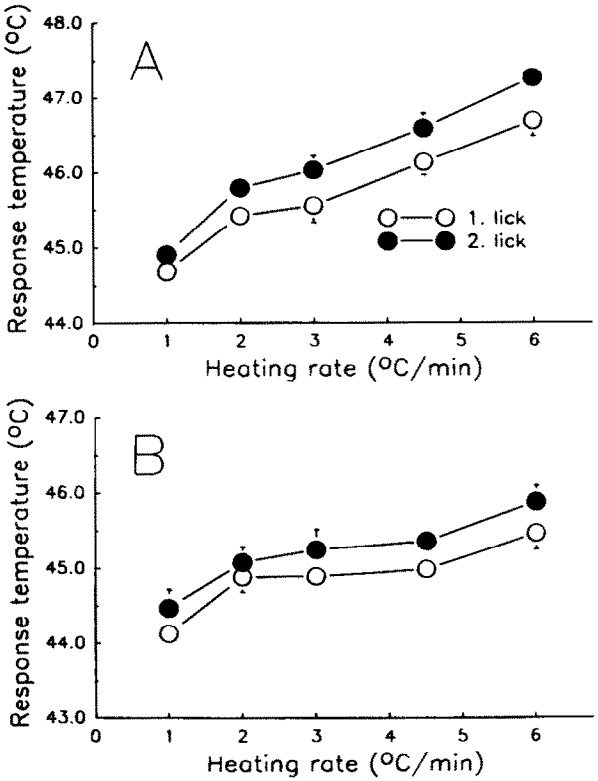


**FIGURE 1. Response temperatures at various heating rates, the first two licking responses (means ± SEM, $n$ = 8 in each group). (A) rats. (B) mice.**

served that the hot plates were contaminated with urine and feces, whereas this was not a problem at heating rates of 3.0°–6.0°C/min.

### Calculation of Nociceptive Temperature Threshold

From these data, it is possible to estimate the temperature threshold for nociception. The first assumption made is that there is a certain delay from the point in time at which the temperature of the plate passes threshold to the time at which the first licking response occurs. This delay is considered to consist of two components, the first being the time required to heat the nociceptors, which lie some distance from the surface of the skin, to threshold (the physical component). The second component is the time from firing of the nociceptors to the occurrence of the recorded behavioral response (the behavioral component). Another assumption made is that the total delay is constant for all heating rates (see Discussion section).

Then we will have for each animal:

$$T_{response} = T_{threshold} + D(dT/dt), \quad (1)$$

where T is the temperature, D is the response delay, and dT/dt the heating rate of the plate.

From Eq. (1) follows:

$$T_{threshold} = T_{response} - D(dT/dt). \quad (2)$$

Hence, the theoretical $T_{threshold}$ for each animal is a function of D. The variance of $T_{threshold}$ will also be a function of D, and an estimate of the true threshold temperature may be found by varying D and minimizing this variance.

In the present experiment, the data for a heating rate of 1.0°C/min were left out of this calculation because of the obvious stress-induced behavior that was expressed during the test. The variance of $T_{threshold}$ was calculated for different values of D (Figure 2), and the value of D was accepted where the variance was minimal. The delay was estimated to 20 sec for rats and 8.8 sec for mice. The threshold temperatures were calculated for each animal according to Eq. (2). Mean threshold temperatures (±SEM) were 44.66° ± 0.092°C for rats and 44.50° ± 0.097°C for mice.

### Evaluation of Tissue Damage

Morphine was administered to the animals in a dose of 10 mg/kg. Groups of rats ($n$ = 4) were tested at a rate of 3.0°C/min to different end temperatures (48.0°, 49.0°, 50.0°, and 51.0°C) and were then immediately removed from the plate. After 24 hr the plantar skin of the hindpaws was visually inspected and compared to control animals. The skin of each hindpaw was given a redness score from 0 to 4. Formation of blisters was noted, each paw with blister(s) was given a redness score of 4 as well as a blister score of 1. Mean redness scores and blister scores were calculated ($n$ = 8 hindpaws).

When the plate was heated to temperatures of 50°C or lower, the paws showed very few blisters. After heating to 51°C, blisters were observed on both hindpaws of all rats on examination the day after exposure to the plate (Figure 3).

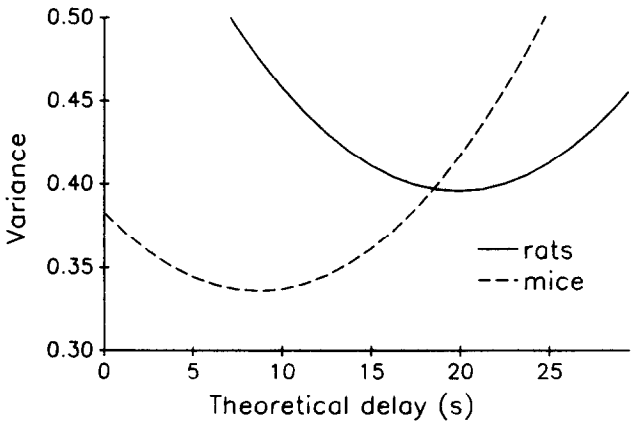


**FIGURE 2. Variance of calculated nociceptive thresholds as a function of delay from threshold to the first licking response.**

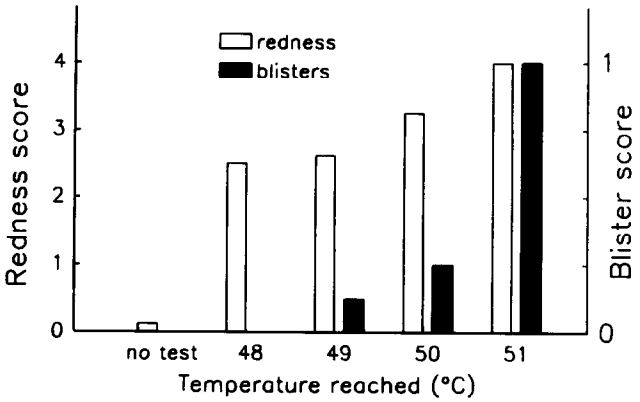


**FIGURE 3. Evaluation of plantar skin damage after heating to various temperatures. Four rats in each group. Mean scores for redness and blisters for all hindpaws ($n$ = 8 paws) in each group. Redness: graded 0–4. No blisters = 0; blister formation = 1.**

One group of mice ($n$ = 8) was exposed to the hot plate at 3.0°C/min up to 50.0°C. After 24 hr no blisters were observed on any paw.

### Influence of Ambient Temperature

Two groups of 10 rats were tested after 2 hr adaptation at ambient temperatures of either 18°C or 28°C. Each group was tested at both ambient temperatures with an interval of 24 hr, according to a crossover design. The tests were performed in the same test room. The response temperatures at these two ambient temperatures were not significantly different (Figure 4).

Mice were tested according to the same protocol as the rats. After 2 hr at an ambient temperature of 28°C, the mice responded at a significantly lower hot-plate

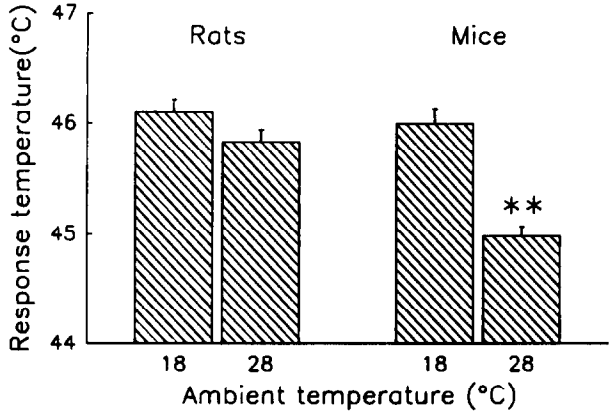


**FIGURE 4. Response temperatures at ambient temperatures of 18° and 28°C. Rats: $n$ = 20 in each group. Mice: $n$ = 16 in each group. Means ± SEM. **$p < 0.001$, $t$ test.**

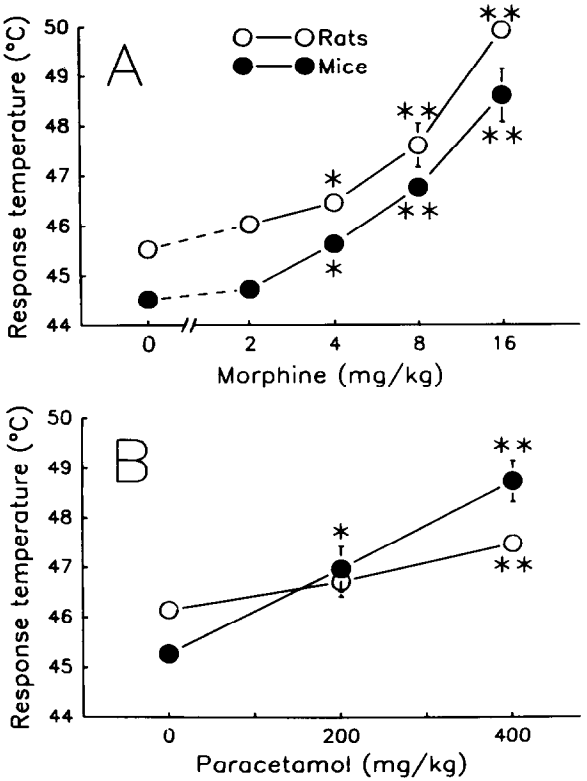


**FIGURE 5. Effects of morphine and paracetamol in rats and mice. (A) morphine, (B) paracetamol ($n$ = 8–12 at each data point. *$p < 0.05$, **$p < 0.01$, compared to vehicle group, $t$ test).**

temperature than did the animals at 18°C, the difference being approximately 1°C [$t(16) = 6.92$, $p < 0.001$, Student's $t$ test, Figure 4].

### Effect of Morphine and Paracetamol

Rats and mice were tested at 30 and at 50 min after injection of morphine and of paracetamol, respectively. A cutoff temperature of 50.0°C was employed. If no hind-paw lick occurred, then the cutoff value was used in the statistical calculations.

Morphine and paracetamol both showed a significant, dose-related antinociceptive effect in the test. (Rats: morphine: $F(4,35) = 41.4$, $p < 0.001$, ANOVA; paracetamol: $F(2,17) = 6.28$, $p < 0.01$, ANOVA. Mice: morphine: $F(4,35) = 30.4$, $p < 0.001$, ANOVA; paracetamol: $F(2,28) = 17.11$, $p < 0.001$, ANOVA; Figure 5).

## DISCUSSION

It has previously been demonstrated that the modified hot-plate test with increasing temperature is a valid and reliable test for nociception, sensitive to mild analgesics (Hunskaar et al., 1986a and b). The apparatus used in the present study

represents an improvement of the test, with better control of the temperature of the plate, the possibility of controlling the rate of temperature rise, and improved testing efficiency. However, some theoretical and practical problems associated with measurement of surface temperatures must be pointed out. The surface temperature of a heated body in contact with the surrounding air is not a well-defined parameter. It is liable to change whenever an animal or a temperature-measuring probe is brought in contact with it. Due to different calibration procedures, there will probably be variations in response temperatures between laboratories, and between different sets of equipment if these are not calibrated according to exactly the same procedure.

These results suggest that the optimal heating rate for nociceptive testing is 3–4°C/min. To minimize the confounding effects of response delays, caused for instance by sensorimotor impairment or sedation, we noted that a slow temperature increase would be preferable. However, rates of 1°C/min and to some extent 2°C/min induced defecation and urination, which may be interpreted as stress responses due to the longer exposure to the hot environment. Heat-dissipating behavior, such as forepaw licking or grooming, which may interfere with the criterion response, may also be more likely to occur when the heating rate is extremely low.

Hindpaw lick has been found to be a reliable criterion of nociception in the hot-plate test (Berge, Fasmer, and Hole, 1983, Hunskaar et al., 1986b). However, hindpaw lick unrelated to noxious stimuli may occur during grooming, resulting in occasional false registrations of nociceptive responses. If this factor were an important source of error, then the variability of the results could be reduced by omitting the first licking response. The response temperatures for the first and second licking period were recorded, and as shown in Figure 1, the variability was not reduced by employing the second licking response rather than the first. Consequently, it seems sufficient to record the first hindpaw lick when performing the test. Testing drugs that influence grooming or motor activity may, we note, however, require that behavior in addition to the first licking period is taken into consideration.

The present method allows estimation of a temperature threshold for nociception. The calculations in the Results section are based on the assumption that the delay from the time at which the plate reaches threshold temperature until the licking response occurs is fairly constant. This is, however, an approximation. The time lag for nociceptors to reach threshold (physical component of delay) will probably vary with changes in heating rate. The situation at the lower heating rates is closer to thermal equilibrium than at the higher rates, and hence, the temperature of the nociceptors is closer to the temperature of the plate. This could lead to a shorter delay for the nociceptors to be heated to threshold. On the other hand, the heat flux from the surface to the depth of the receptors is less, and it would therefore be difficult to make a reliable prediction of the influence of the rate of temperature increase upon the physical component of the delay.

A high rate of heating may be expected to produce a steeper increase in the firing-rate of the nociceptors, and may hence cause a shorter behavioral component in the delay. However, at all rates used in this experiment, the increase in temperature during the calculated delay is small.

In view of these considerations, we found it acceptable to assume an approximately constant delay. The calculations lead to very similar thresholds for rats and mice at the nociceptor level, and this supports the assumptions made before calculation. The calculated thresholds of approximately 44.5°C are similar to previous findings of dorsal horn neurons in rats having thresholds of 42.5°–45°C for tail stimulation (Necker, 1978), and somewhat higher than a temperature threshold for rat tail-flicks found to be 42.6°C at the depth of the nociceptors (Ness and Gebhart, 1986).

The calculated delay is longer in rats than in mice, 20 versus 8.8 sec. This may be due to the difference in skin thickness in the two species, with shorter distance to the nociceptors in mice, which should lead to a shorter physical component of the delay. In addition, the behavioral component may differ, possibly with longer behavioral delay in rats, which seem to show a more complex pattern of nociceptive behavior on the hot plate.

It is important to minimize pain and suffering in experimental animals (Zimmermann, 1983). The investigation of tissue damage in this study was considered essential to minimize suffering of the animals in later experiments. In the increasing-temperature hot-plate test, an appropriate response terminates the stimulus, and the animals are exposed to pain to a limited extent. Control animals invariably respond at temperatures well below 50°C, and hence never show blistering. The temperature interval between normal response temperatures and the recommended cutoff temperature of 50°C is sufficiently large to allow adequate separation between doses of analgesics. Thus, the test can be performed without inducing tissue damage. If higher cutoff temperatures than 50°C are considered necessary, then it may be advisable to sacrifice the animals before the effects of the analgesic wane, in order to prevent suffering. Besides being positive from the ethical point of view, it is essential for performing repeated tests that the procedure does not inflict tissue damage.

Recently, it has been shown that the skin temperature is an important factor in determining the results of the tail flick test, another nociceptive test with a thermal stimulus (Berge, Garcia-Cabrera, and Hole, 1988; Tjølsen, Lund, Berge, and Hole, 1988; Eide, and Tjølsen, 1988). Hence, the possible effect of changes in ambient temperature on the increasing-temperature hot-plate test was investigated. There was no significant difference in the results between rats kept at 18° and 28°C for 2 hr. Mice, however, showed a significant difference in response temperatures, with higher response temperatures after cold exposure.

This effect may be due to lower peripheral skin temperature in a cool environment, causing a slower heating of the nociceptors to reaction threshold, and a prolonged physical component of the delay. When housed individually in a cool environment, the animals lack the opportunity to prevent heat loss by huddling. Mice, with a large surface-to-volume ratio, may have difficulties keeping their body temperature at a normal level, and may be exposed to a considerable cold stress. In this heat-conserving state, the peripheral blood flow is reduced and the skin temperature is lowered, reducing heat loss.

Antinociceptive effects were demonstrated for both morphine and paracetamol,

although the maximal effect obtainable for paracetamol in rats was considerably smaller than for morphine. The results confirm that the increasing-temperature hot-plate test is suitable for showing antinociceptive effects of the weak, nonopioid analgesics.

In conclusion, the increasing-temperature hot-plate test has been better characterized, using an improved test apparatus that provides ease of testing and better control over stimulus variables. For most purposes a rate of temperature increase of 3.0°C/min and a cutoff temperature of 50°C provide adequate separation between analgesic effects of various treatments, without exposing the animals to tissue damage.

Diagrams of the electronic circuitry of the equipment and program listings are obtainable from the authors upon request.

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